Influence of Chemotherapeutic Drugs on Human Granulosa Cells

Dissertation

In partial fulfillment of the requirements for the academic degree of

doctor rerum naturalium (Dr. rer. nat.)

submitted to the Faculty Council of the School of Medicine at Friedrich Schiller University of Jena

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Date of the public disputation: 29.08.2017

This work is dedicated…

to my beloved husband

to my mother

to my family for their endless love, support and encouragement.

List of Abbreviations

ALL	Acute lymphoblastic leukemia
AMH	Anti-Müllerian Hormone
BMP15	Bone morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
CHOP	Vincristine, cyclophosphamide, doxorubicin and prednisone
CYC	Cyclophosphamide
CYP11A1	Cytochrome-P450 cholesterol side-chain cleavage
CYP17A1	Enzyme 17α-hydorxylase-17, 20-desmolase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DHEA	Dehydroepiandrosterone
DHFR	Dihydrofolate reductase
DOX	Doxorubicin
dNTPs	Deoxynucleotide triphosphates
E2	Estradiol
FBS	Fetal bovine serum
FF	Follicular fluid
FSH	Follicle stimulating hormone
GDF9	Growth-differentiation factor 9
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IVF	In vitro Fertilisation
LH	Luteinizing hormone
miRNAs	MicroRNAs
miR-21	MicroRNA-21
miR-132	MicroRNA-132
MTD	Maximum tolerated dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H tetrazolium)
MTX	Methotrexate
NADP	Nicotinamide adenine dinucleotide phosphate
NHL	Non-Hodgkin lymphoma
OD	Optical density
OSFs	Oocyte-secreted factors
PBS	Phosphatase buffered saline
PCOS	Polycystic ovary syndrome
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3 kinase
POF	Premature ovarian failure
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNU48	Small nucleolar RNA U48
RT	Reverse transcription

SD	Standard Deviation
SE	Standard Error
TGF-*b*	Transforming growth factor-beta
VCR	Vincristine

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Summary

Cancer is one of the leading causes of death in persons aged 1 to 14 years and leukemias are the most common malignancies in childhood and are the primary cause of cancer-related mortality in the world. Chemotherapy is quite effective in treating cancers, but after successful treatment, young female cancer survivors face a potential threat of premature ovarian failure (POF) and infertility from gonadotoxicity of chemotherapeutic agent. Cytostatic drugs can target the oocyte directly, or can induce oocyte death indirectly via damage to surrounding granulosa cells, which play a key role in folliculogenesis and oocyte maturation. A more thorough understanding of the mechanism behind chemotherapy-induced infertility is necessary to develop new methods to preserve fertility in these patients. MicroRNAs (miRNAs) have recently begun to be explored in ovarian cells as small molecules with critical regulatory role. Importantly, the miRNAs profile in granulosa cells may possess high potential as a new marker for successful folliculogenesis and oocyte development. They can control steroidogenesis in cultured granulosa cells and are involved in control of proliferation, apoptosis and carcinogenesis. MicroRNA-21 (miR-21) and microRNA-132 (miR-132) are of the most abundant and highly expressed miRNAs in human oocytes and granulosa cells. miR-21 is an oncogenic miRNA and plays a critical role in maintaining the survival of granulosa cells in periovulatory follicles and acts as an antiapoptotic factor in granulosa cells. miR-132 has a key role in promotion of estradiol (E2) synthesis in murine ovarian granulosa cells.

The present study aimed to determine in vitro the potential association of different chemotherapeutic agents as part of childhood cancer treatment with infertility in adults who survived pediatric cancer and to explore effects of chemotherapeutic agents on expression of two important miRNAs (miR-21 and miR-132) in granulosa cells. The concentration-dependent cytotoxicity of doxorubicin (DOX), vincristine (VCR), methotrexate (MTX) and cyclophosphamide (CYC) has been assessed against isolated granulosa cells from women receiving assisted reproduction treatment and human granulosa cell lines (KGN and COV434) in comparison to human leukemia T cell line Jurkat. The granulosa cells were cultured for 72 h and then incubated with different doses of doxorubicin (0.01, 0.05, 0.1, 0.2 and 0.5 μg/ml), vincristine (0.01, 0.1, 1, 5 and 10 μg/ml), methotrexate (0.1, 1, 5, 10 and 50 μg/ml) or cyclophosphamide (1, 5, 10, 50 and 100 μg/ml) for 48 h. Simultaneously, the granulosa cell lines (KGN and COV434) and Jurkat cells were treated for 12, 24 and 48 h with the same doses of doxorubicin, vincristine, methotrexate or cyclophosphamide. Subsequently, cell

viability was evaluated by MTS assay and apoptosis by annexin V and propidium iodide staining in flow cytometry and the concentrations of estradiol in culture supernatants by immunoassay. Effects of chemotherapeutic agents on expression miR-21 and miR-132 in primary granulosa cells and cell lines as well as in Jurkat cells were assessed by using quantitative real time-PCR.

The results showed that the drugs induced time- and concentration-dependent inhibitory effects on the proliferation of primary granulosa cells and cell lines and different levels of apoptosis. Cyclophosphamide and doxorubicin exerted a similar degree of cytotoxicty on primary granulosa cells and resulted in significant decrease in cell viability, increased cell death and decreased estradiol concentration linearly. Treatment with methotrexate induced cell death in Jurkat cells, while the magnitude of this response in primary granulosa cells was significantly less pronounced. Vincristine had the highest cytotoxic effect on viability of Jurkat, KGN and COV434 cells. Interestingly, doxorubicin induced overexpression of miR-21 in COV434 and miR-132 in KGN and Jurkat cells.

In conclusion, these findings demonstrate the extremely damaging effects of cyclophosphamide and doxorubicin on granulosa cells and the comparatively low influence of methotrexate and vincristine on the viability and steroidogenesis of human primary granulosa cells. This may suggest, at similar anti-leukemia efficacy of the compared drugs, that methotrexate and vincristine induce lower ovarian and reproductive toxicity. This study may provide novel information on the effects of doxorubicin on expression of miR-21 and miR-132 which may bring benefits to reproductive medicine.

Zusammenfassung

Die Erkrankung Krebs stellt eine häufigste Todesursache bei Personen im Alter von 1 bis 14 Jahren dar. Die Leukämie zählt hierbei als die häufigste Krebserkrankung im Kindesalter und gilt darüber hinaus als weltweit primäre Ursache der Krebsmortalität. Zur Behandlung von infantilen Krebserkrankungen wird die Chemotherapie als effektive Option eingestuft, welche jedoch das Risiko birgt, dass nach erfolgreicher Behandlung junge weibliche Krebsüberlebende aufgrund der Gonadotoxizität von Chemotherapeutika vorzeitig von Ovarialinsuffizienz (POF) sowie Infertilität betroffen sind. Viele Zytostatika richten sich direkt auf die Oozyte oder können den Oozytentod indirekt durch Schädigung der umgebenden Granulosazellen induzieren, welche eine Schlüsselrolle für die Follikulogenese und die Oozytenreifung spielen. Ein gründlicheres Verständnis des Mechanismus der Chemotherapie-induzierten Infertilität ist notwendig, um neue Methoden zur Erhaltung der Fertilität dieser Patienten zu entwickeln.

In den vergangenen Jahren wurden mikroRNAs (miRNAs) als kleine Moleküle mit bedeutender regulatorischer Rolle in Ovarialzellen eingestuft. Dabei besitzt das miRNA-Profil in Granulosazellen ein hohes Potential, als diagnostischer Marker für eine erfolgreiche Follikel- und Oozytenentwicklung genutzt werden zu können. MiRNAs können die Steroidogenese in kultivierten Granulosazellen kontrollieren und beeinflussen regulatorische Prozesse wie Proliferation, Apoptose und Karzinogenese. miRNA-21 (miR-21) und miRNA-132 (miR-132) sind die am stärksten exprimierten miRNAs in menschlichen Oozyten und Granulosazellen. Die onkogene miR-21 spielt eine wichtige Rolle bei der Aufrechterhaltung des Überlebens von Granulosazellen in periovulatorischen Follikeln und wirkt als anti-apoptotischer Faktor in Granulosazellen. miR-132 besitzt eine Schlüsselrolle bei der Förderung der Östradiol-Synthese in murinen Ovarialgranulosazellen.

Ziel der vorliegenden Studie war es, den möglichen Zusammenhang zwischen der einer erfolgten Chemotherapie im Kindesalter und einer verstärkt auftretenden Infertilität bei jungen Erwachsenen, die pädiatrische Krebserkrankungen überlebt haben, in vitro zu untersuchen. Des Weiteren sollte der Einfluss dieser Chemotherapeutika auf die Expression von zwei für die Regulation von Granulosazellen wichtigen miRNAs (miR-21 und miR-132) erforscht werden. Die konzentrationsabhängige Zytotoxizität von Doxorubicin (DOX), Vincristin (VCR), Methotrexat (MTX) und Cyclophosphamid (CYC) auf primäre Granulosazellen, isoliert von der Follikelflüssigkeit von IVF-Patientinnen sowie auf zwei etablierte humane Granulosazelllinien (KGN und COV434) wurde analysiert und mit den Untersuchungen zytostatischer Wirkungen auf die humane leukämische T-Zelllinie Jurkat

gegenübergestellt. Dazu wurden die Granulosazellen für 72 h kultiviert und anschließend mit Doxorubicin (0,01, 0,05, 0,1, 0,2 und 0,5 µg/ml), Vincristin (0,01, 0,1, 1, 5 und 10 µg/ml), Methotrexat (0,1, 1, 5, 10 und 50 µg/ml) oder Cyclophosphamid (1, 5, 10, 50 und 100 µg/ml) in 5 verschiedenen Konzentrationen für 48 Stunden inkubiert. Die beiden Granulosazelllinien (KGN und COV434) sowie die Jurkat-Zellen wurden ebenfalls für 12, 24 und 48 h mit denselben Dosen von Doxorubicin, Vincristin, Methotrexat oder Cyclophosphamid behandelt. Anschließend wurde die Zellvitalität mittels MTS-Assay bestimmt, während der Anteil an apoptotischen Zellen durch die Annexin-V-und Propidiumiodid-Färbung via Durchflusszytometrie bewertet wurde. Gleichzeitig wurde die Konzentrationen von Östradiol in Kulturüberständen durch Immunoassays ermittelt. Die Effekte der Chemotherapeutika auf die Expression von miR-21 und miR-132 in primären Granulosazellen, den Granulosa-Zelllinien sowie in Jurkat-Zellen wurde mittels quantitativer real time-PCR untersucht.

Die Ergebnisse zeigten, dass die untersuchten Medikamente Zeit- und konzentrationsabhängige inhibitorische Effekte auf die Proliferation von primären Granulosazellen und die beiden Granulosa-Zelllinien hervorrufen und verschiedene Apoptoseraten induzieren. Cyclophosphamid und Doxorubicin wirkten auf primäre Granulosazellen ähnlich zytotoxisch, führten zu einer signifikanten Abnahme der Zell viabilität, einem erhöhten Zelltod und einer linear abnehmenden Östradiolkonzentration. Die Behandlung mit Methotrexat induzierte den nahezu vollständigen Zelltod in Jurkat-Zellen, während in primären Granulosazellen signifikant weniger Zellen apoptotisch reagierten. Vincristin zeigte die höchste zytotoxische Wirkung auf die Zellviabilität von Jurkat, KGN und COV434 Zellen. Interessanterweise induzierte Doxorubicin eine Überexpression von miR-21 in COV434 und miR-132 in KGN und Jurkat-Zellen.

Zusammenfassend zeigten diese Untersuchungen die äußerst schädigende Wirkungen von Cyclophosphamid und Doxorubicin auf die Granulosazellen und einen vergleichsweise geringen Einfluss von Methotrexat und Vincristin auf die Zellviabilität und Steroidogenese der humanen primären Granulosazellen. Dies kann bei einem zugleich ähnlichen anti-leukämischen Effekt der untersuchten Medikamente nahelegen, dass Methotrexat und Vincristin die geringste Eierstock- und Reproduktionstoxizität induzieren. Zugleich konnten neue Erkenntnisse über die Wirkung von Doxorubicin auf die Expression von miR-21 und miR-132 generiert werden, was für den Bereich der reproduktiven Medizin in Zukunft genutzt werden könnte.

CHAPTER 1 INTRODUCTION

1. Overview of the ovary

The mammalian ovary is a complex organ and has a variety of functions essential to the reproductive process. The human ovary by birth contains a fixed number of germ cells (oocytes) within primordial follicles, a basic functional unit which consists of an immature oocyte in meiotic arrest, enclosed by a small number of flattened pregranulosa cells. The primordial follicles constitute the resting pool of female germ cells required for the reproductive lifespan of the female (Edson et al. 2009). At around five months of gestational age, the human ovary establishes several million primordial follicles which are followed by a decline to approximately 1-2 million at the time of birth, about 300,000 at puberty and approximately 1,000 at an average age of 50–51 years, defined as the onset of the menopause. In the normal human reproductive life span, there are around 450 menstrual cycles. In each cycle small cohorts of primordial follicles are recruited out of the resting primordial pool and are activated to grow (Wallace and Kelsey 2010). Abnormalities in primordial follicle development lead to a number of pathophysiologies like premature ovarian failure and female infertility (Skinner 2005).

1.1. Formation of ovarian follicle

The ovarian follicle is the basic functional unit in the ovary that is composed of a developing oocyte and somatic cells. The granulosa cells and theca cells are the two primary somatic cell types in the ovarian follicle (Edson et al. 2009). Follicle formation is a process that known as germ cell nest breakdown. During fetal development, primordial germ cells (oocyte precursors) migrate from the yolk sac to the genital ridge where the undifferentiated gonad resides (Tingen et al. 2009). These germ cells (oogonia) massively proliferate by mitosis and develop in clusters or nests in which oogonia surrounded by squamous pre-granulosa cells (Gondos 1973). When germ cell nest formation is complete, mitosis of oogonia is ceased and meiosis starts. It is here that the oogonia become oocytes; thereafter these oocytes are arrested in prophase I of meiosis (Borum 1961).

1.2. Folliculogenesis

Development and maturation of follicles to ovulation of the oocyte for subsequent fertilization, a process termed folliculogenesis, is one of the primary functions of the ovary (Edson et al. 2009). After puberty and through an unknown selection mechanism, small cohorts of primordial follicles are recruited out of the resting pool to undergo growth and differentiation (McGee and Hsueh 2000). However, normally only one follicle fully matures, and the rest undergo atresia, which is follicular programmed cell death via apoptosis. Once activated, the surrounding granulosa cells of these primordial follicles change from a flat to a cuboidal structure, marking the beginning of the primary follicle (Fig. 1). In the primary follicle, the oocyte grows and granulosa cells proliferate and form multiple layers of somatic cells that surround the oocyte, resulting in the formation of a secondary (preantral) follicle. After formation of the secondary follicle, theca cells begin to appear and surround the granulosa cells by formation of a layer (Matsuda et al. 2012). Then fluid filled patches form in the granulosa cell layers within the follicle, which coalesces to form the early antral (or tertiary) follicle (Soyal et al. 2000).

Follicles at this stage are gonadotropin-responsive and begin to synthesize sex steroid hormones, because of the presence of both granulosa and theca cells. Pituitary follicle stimulating hormone (FSH) stimulates the follicle for further growth (Fritz and Speroff 2011) and the antrum continues to enlarge by action of FSH, resulting in the formation of a preovulatory follicle. The oocyte within the preovulatory follicle is surrounded by cumulus cells and as the preovulatory follicle continues to mature and increase in size, due to an increased volume of follicular fluid, granulosa cells produce estradiol. Thereafter, the luteinizing hormone (LH) surges causing the follicle to ovulate (Kumar and Sait 2011). When the oocyte is released, granulosa and theca cells differentiate into luteinized cells, to form the corpus luteum (Georges et al. 2014).

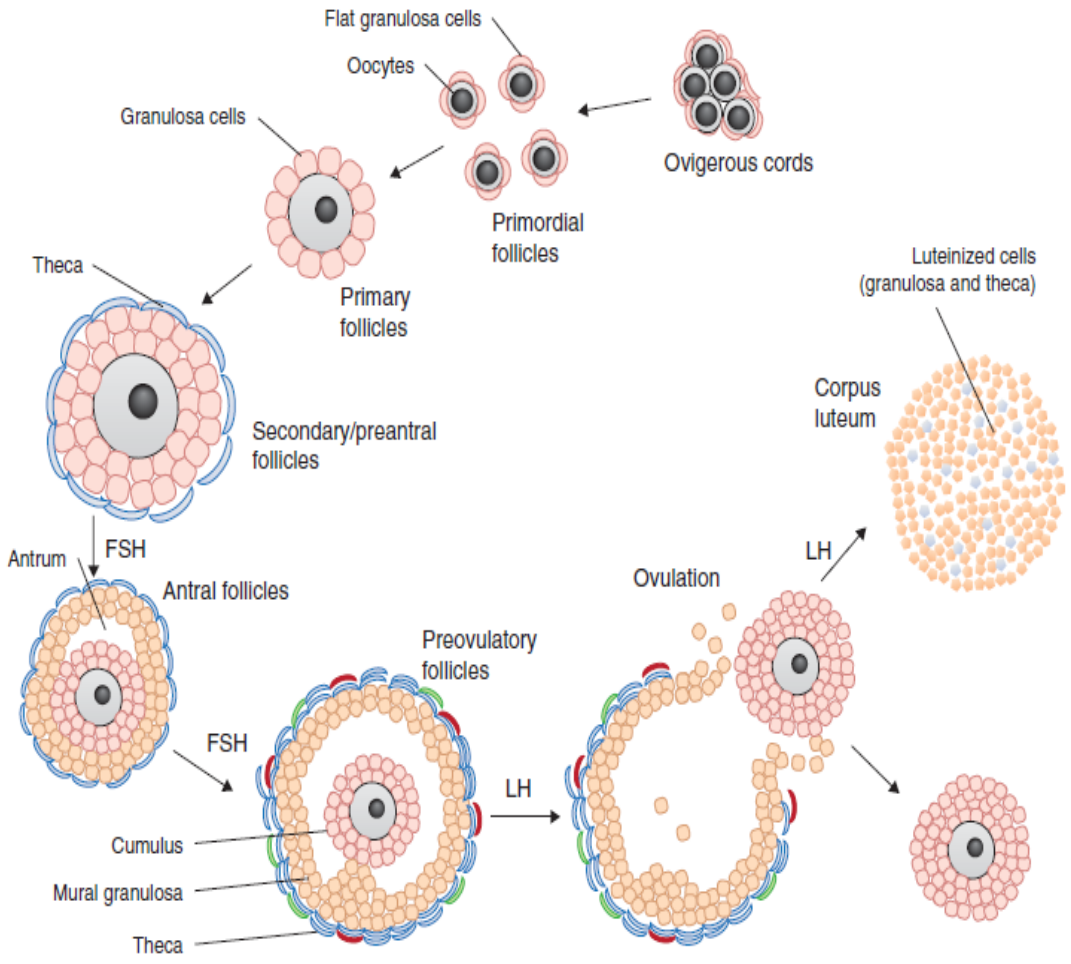


Figure 1. Ovarian folliculogenesis
Folliculogenesis begins with the recruitment of primordial follicles from a reserve pool to become primary follicles. Granulosa cells proliferate and form multiple layers around the oocyte; theca cells form a layer around the follicle in secondary (preantral) follicles. (FSH) stimulates the follicle for further growth to form antral and subsequently to preovulatory follicle. After ovulation, the antral follicle differentiates into the corpus luteum, and the granulosa and theca cells become luteinized cells. Modified (Georges et al. 2014).

1.3. Ovarian granulosa cells

The most important cell type in the ovary that function as the ovarian nurse cells is granulosa cells which support development of the oocyte physically and provide the required microenvironment. Granulosa cells are somatic cells surrounding closely the oocyte and actively differentiating cells (Edson et al. 2009). Proliferation and cellular differentiation of granulosa cells are critical aspects for normal follicular growth, ovulation and luteinisation. During folliculogenesis, granulosa cells undergo alteration in cellular differentiation. Numerous actions of hormones and growth factors are required for regulation of granulosa cell differentiation. In the early primordial follicle stage, cells are non-steroidogenic and independent on steroid hormone (Richards and Midgley 1976, Oktay et al. 1997). However, in developmental stages before ovulation, FSH stimulates granulosa cells to convert androgens (coming from the thecal cells) to estradiol via cytochrome P450 aromatase (Albertini et al. 2001, Senthilkumaran et al. 2004). After ovulation, granulosa cells turn into granulosa lutein cells that produce progesterone. The progesterone may maintain a potential pregnancy. The biosynthesis of two important steroid hormones in ovary, estradiol and progesterone, is a primary function of the granulosa cells in human. Estradiol plays an important role in ovarian follicle development, oocyte maturation, endometrial proliferation and its dysregulation contributes to the development of polycystic ovary syndrome (PCOS) and premature ovarian failure (Drummond and Findlay 1999). Granulosa cell possess specific receptors for the gonadotropins FSH and LH (Richards und Midgley 1976), for growth factors such as insulin-like growth factor (IGF) (Adashi 1998) and anti-Müllerian Hormone (AMH), a member of the transforming growth factor beta (TGF-*b*) superfamily of growth factors (Knight and Glister 2006).

Granulosa cells have an important role in keeping of oocyte quality, which is a key factor in female fertility. The bidirectional communication between oocyte and granulosa cells is essential and must be tightly coordinated. The oocyte interacts with the granulosa cells through gap junctions which are channels formed by connexin proteins. However, oocytes have a closely control their surrounding somatic cells and directing them to perform functions required for correct development of the oocyte. Oocyte secretes growth-differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), as two important oocyte-secreted factors (OSFs) which are members of the TGF-*b* superfamily, which activate signalling pathways in granulosa cells to regulate key genes and cellular processes required for their differentiation. In turn, granulosa cells are responsible for promoting oocyte growth and

development through ovarian follicular microenvironment and maternal signals (Gilchrist et al. 2008).

1.3.1. Heterogeneity of granulosa cells

The granulosa cells differentiate to become two anatomically and functionally distinct populations upon antrum formation (Fig. 2) (Telfer et al. 1988). Cumulus granulosa cells surround the oocyte and are ovulated with it. Through gap junctions between cumulus cells and the oocyte, cumulus cells provide nutrition for the oocyte and influence oocyte development as well as keeping the oocyte in meiotic arrest (Eppig und Downs 1984, Khamsi et al. 2001). The mural granulosa cells, which line the follicle wall and border the theca cells and play a steroidogenic role, remain behind following ovulation and regress to form the corpus luteum. Additionally, cumulus granulosa cells are different from mural granulosa cells in their response to gonadotropins (Khamsi et al. 2001).

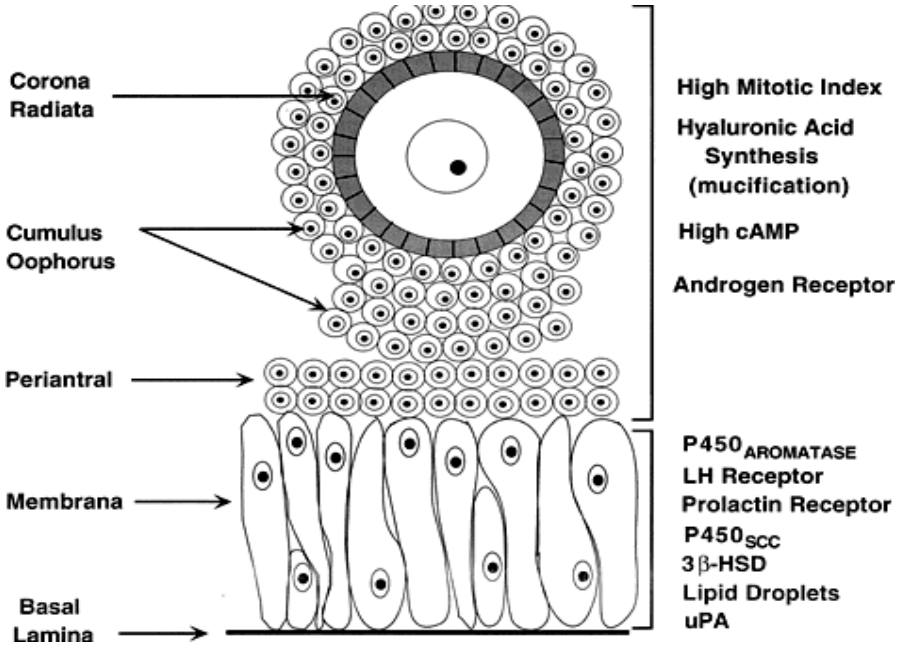


Figure 2. Heterogeneity of the granulosa cellsin a healthy Graafian follicle.
The cumulus granulosa cells surround the oocyte and mural granulosa cells line the follicle. Modified (Erickson 2008).

1.4. Ovarian Steroidogenesis

Another primary function of the ovary is steroidogenesis which is a tightly regulated and complex process that involves a number of enzymatic reactions in both the theca and granulosa cells (Fig. 3). Steroidogenesis can be described with the two cell/two gonadotropin model (Hillier et al. 1994). In the early antral follicle theca cells contain LH receptors and, after stimulation, cholesterol is converted to pregnenolone by action of cytochrome-P450 cholesterol side-chain cleavage (CYP11A1) (Miller 1988, Hanukoglu 1992). The enzyme 17α-hydroxylase-17,20-desmolase (CYP17A1) converts pregnenolone and progesterone to dehydroepiandrosterone (DHEA) and androstenedione, respectively. In contrast to theca cells, granulosa cells of the early antral follicle contain only FSH receptor, which are reliant on androstenedione produced by the surrounding thecal cells to produce estradiol by action of aromatase (Jamnongjit and Hammes 2006).

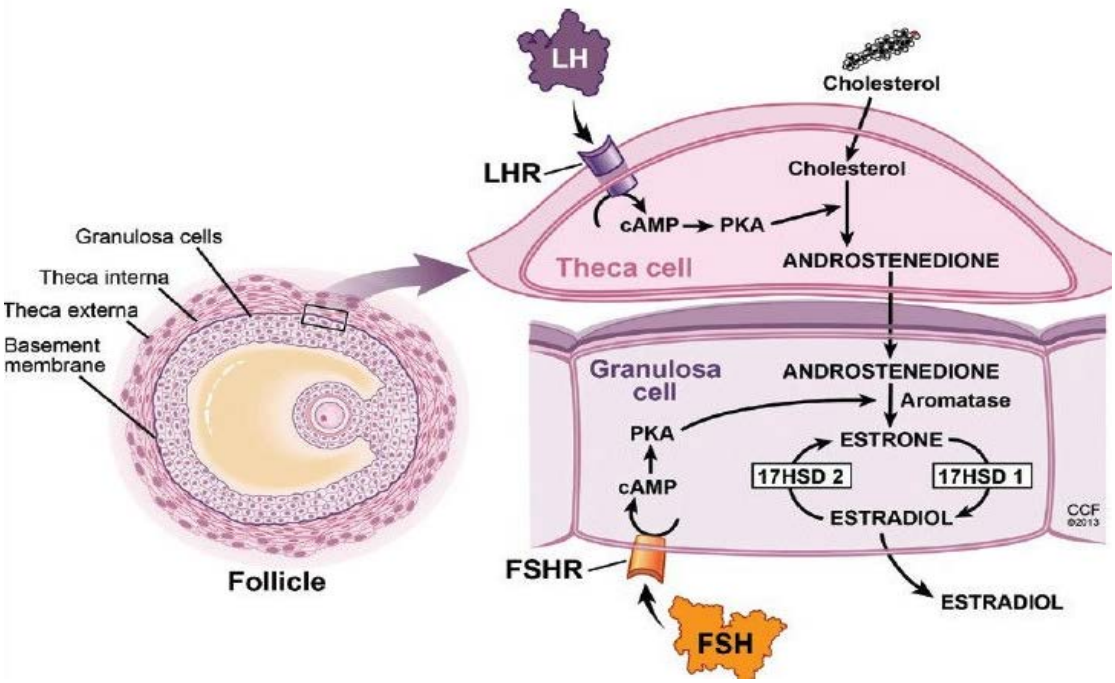


Figure 3. Two-cell, two-gonadotropin model of regulation of estrogen synthesis in the human ovary.

Theca cells produce androstenedione from cholesterol after LH stimulation. Androstenedione is then transported to the granulosa cells where it is aromatized to estrone and finally converted to estradiol after FSH stimulation. Modified (Doshi and Agarwal 2013).

2. Common childhood cancers

Childhood cancer is one of the leading causes of death by disease among children in the world and the number of reported new cancer cases is increasing every year. The most common types of cancer diagnosed in children are acute lymphoblastic leukemia ALL (26%), brain and central nervous system tumors (21%), neuroblastoma (7%) and non-Hodgkin lymphoma (NHL) (6%) (Fig. 4) (Ward et al. 2014). Currently, treatment of childhood cancer has become increasingly successful, where approximately 80% of all patients diagnosed before 15 years of age will survive for 5 years (Keegan et al. 2016). The current number of survivors within Europe is unknown but is estimated to be between 300,000–500,000, which in Western Europe translates to one in 750 young adults being survivors of childhood cancer (Stiller et al. 2006).

Successful treatment of childhood cancers is achieved through multimodal approaches involving combination chemotherapy, radiotherapy and surgery. With exception of surgery, these treatments can adversely impact reproductive organs (Metzger et al. 2013). Ovaries are at risk to cancer treatment because of the finite numbers of non-renewable germ cells (Wo and Viswanathan 2009).

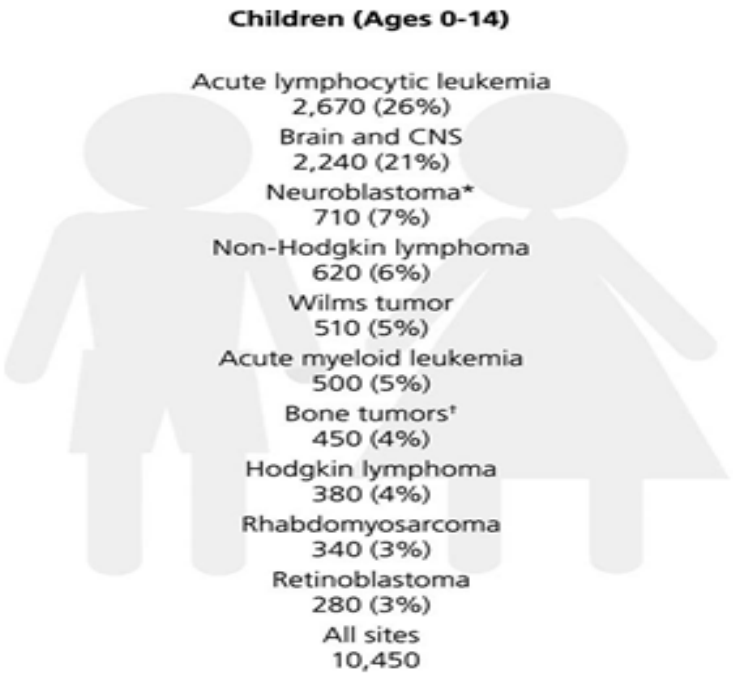


Figure 4. Estimated New Cases of Childhood Cancers United States, 2014.
Malignant cancers are onlyestimates and additionally 730 children will be diagnosed with benign andborderline brain tumors in 2014. *Includes ganglioneuroblastoma. †Bone tumors include osteosarcoma and Ewing sarcoma. Modified (Ward et al. 2014).

3. Chemotherapy drugs

Chemotherapy drugs are cytotoxic agents that used at their maximum tolerated dose (MTD) on the basis that this results in a large damage on cancer cells (Kummar et al. 2006). Cancer patients are treated dependently on each disease by combination chemotherapy protocols including chemotherapy agents, doses and duration of treatment (Meirow 2000). There are many different chemotherapy agents that are commonly used in the treatment of childhood cancer (Bickert 2002). For the present work, four different categories of chemotherapy agents (doxorubicin, vincristine, methotrexate and cyclophosphamide) have been chosen whose mechanisms of action and gonadotoxic potential are different.

3.1. Doxorubicin

Doxorubicin is an anthracycline antibiotic and one of the most commonly used chemotherapeutic drugs. It has been used to treat breast cancer, childhood solid tumors, leukemia and soft tissue sarcomas since the 1960's (Minotti et al. 2004). As it plays a key role in the treatment of many types of cancer, its toxicity on noncancerous cells in the human body has been widely investigated. There is evidence that doxorubicin can cause damage in healthy tissue in brain, heart, liver and kidney by induction of apoptosis and necrosis (Tacar et al. 2013).

The mechanism of doxorubicin in cancer cells includes many different actions (Gewirtz 1999). Doxorubicin enters the cell via passive diffusion and it can intercalate the base pairs of the DNA's double helix, with inhibition of both DNA and RNA polymerase, eventually stopping DNA replication and RNA transcription. The major action of doxorubicin is inhibition of DNA associated enzymes such as topoisomerase enzymes I and II, which are the main targets of doxorubicin, resulting in DNA damage by induction of programmed cell death (Tacar et al. 2013). Topoisomerases I are enzymes that cut the phosphate backbone one of the two strands of double-stranded DNA, while topoisomerase II cut both strands of the DNA helix and both allow for the unwinding of DNA (Press et al. 2011). Generation of free radicals is another action of doxorubicin which leads to DNA damage, inhibition of macromolecule production, DNA unwinding and an increase in alkylation (Gewirtz 1999, Minotti et al. 2004).

The risk of doxorubicin on fertility has been categorized as medium (Wallace et al. 2005). After treatment with doxorubicin-containing protocols, the risk of amenorrhea ranges from 20% to 80% and is dependent on the women's age (Knobf 2006, Lee et al. 2006, Ben-Aharon et al. 2010). In an early study, amenorrhea was reported by 80% of premenopausal women

treated with chemotherapy regimens containing doxorubicin and most women over 40 years of age had irreversible amenorrhea, while 50% of women under 40 years of age had reversible amenorrhea (Hortobagyi et al. 1986).

3.2. Vincristine

Vincristine is an antimicrotubule agent, belonging to the class of Vinca alkaloids and cell cycle–specific cytotoxic drug. It was first isolated in 1961 (Raviña 2011) and used as a therapy for various cancers including breast cancer, Hodgkin's disease, multiple myeloma, and lung cancer. Vincristine is effective in the treatment of acute lymphoblastic leukemia (Ong et al. 2008).

Vincristine's mode of action as microtubule-targeted drug is suppression of microtubule dynamics by binding to the β subunit of the αβ–tubulin heterodimer and thereby disrupting microtubule structures of the cell cytoskeleton and mitotic spindle. Microtubules are major structural components of the cytoskeleton and play a key role in mitosis and are the target of a large and diverse group of anticancer drugs. Destabilization of microtubules by vincristine leads to mitotic block at the metaphase and apoptosis (Jordan 2002, Jordan and Wilson 2004).

The risk of vincristine on fertility has been categorized as low (Wallace et al. 2005). Vincristine is usually applied in combination treatment with other cytostatic drugs. In treatment of Hodgkin's disease, a combination of vincristine, cyclophosphamide, procarbazine hydrochloride and prednisone, given in six cycles for 6 months, increased the response rate and survival of patients (Devita et al. 1970). The ovarian samples from patients who received alkylating regimen CHOP (vincristine, cyclophosphamide, doxorubicin and prednisone) had a decrease in primordial follicle counts and diminished follicle density when compared with untreated controls (Oktem and Oktay 2007a).

3.3. Methotrexate

Methotrexate is an antimetabolite and was first identified as an anti-tumour agent in the 1947 and being less toxic than other available agents (Sneader 2005). It is a powerful agent in treatment of acute lymphoblastic leukemia and it also used to treat choriocarcinoma, lymphoma (Bertino 1993), breast cancer (Tanabe 2016) and as well as rheumatoid arthritis (Lopez-Olivo et al. 2014, Kivitz et al. 2016) and ectopic pregnancy (Guth et al. 2011), either alone or in combination with other agents. As an antifolate methotrexate reduces the folic acid concentration thus leading to decreased leukemia cell populations in the blood (Bertino 1993). On the other hand, methotrexate can cause clinical neurotoxicity and asymptomatic leukoencephalopathy in children (Bhojwani et al. 2014).

Mechanism of action of methotrexate in cancer cells is competitive inhibition of dihydrofolate reductase (DHFR), which is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid in presence of nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor. Tetrahydrofolate is essential for the biosynthesis of thymidylate, de novo purine biosynthesis and several amino acid metabolisms. Because of its metabolic importance, DHFR has been a target for methotrexate. Therefore, inhibition folic acid that is required in nucleotides biosynthesis leads to inhibition of DNA, RNA and proteins production and eventually resulting in cell death (Fig. 5) (Rajagopalan et al. 2002).

Estimated risk of gonadal dysfunction with methotrexate is low risk (Wallace et al. 2005) and some studies reported no effect on fertility in women treated with methotrexate for ectopic pregnancy (Oriol et al. 2008, Uyar et al. 2013). In contrast, another study observed reduced endometrial glands and ovarian follicles in uteri and ovaries of mice treated with methotrexate for 6 months (Chelab and Majeed 2009).

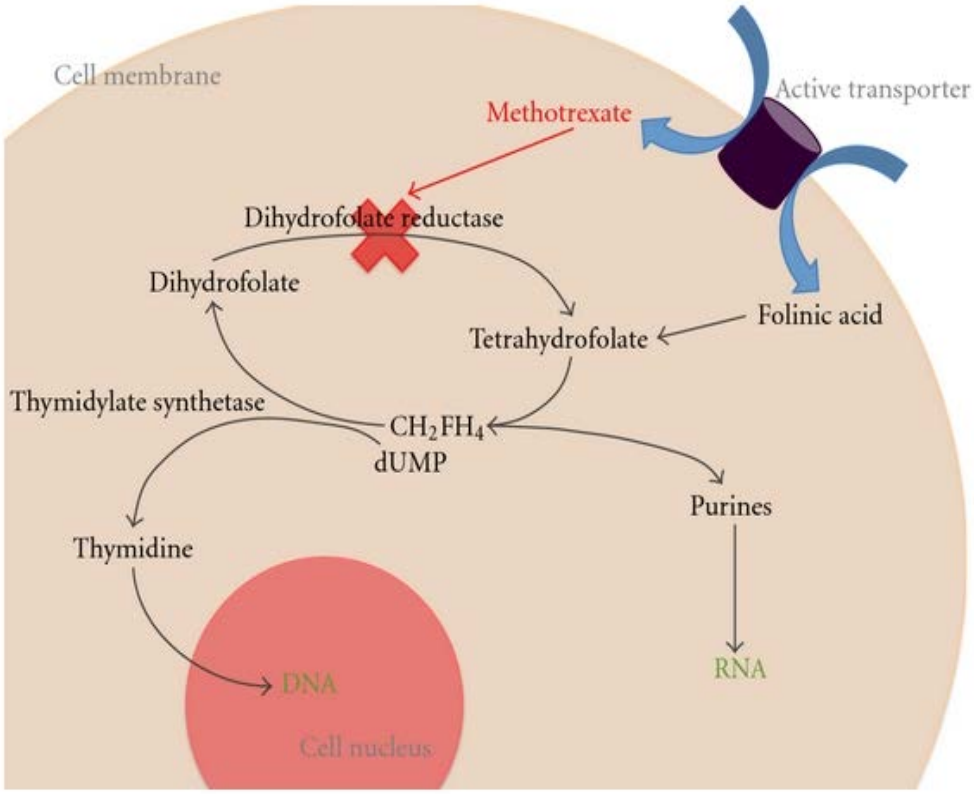


Figure 5. Methotrexate mechanism of action
Methotrexate enters the cell through the reduced folate carrier using an endocytic pathway activated by a folate receptor. After entering the cell, methotrexate inhibits the enzyme dihydrofolate reductase, thus blocking the conversion of dihydrofolate to tetrahydrofolate. As result, thymidine and purine biosynthesis is reduced, this ultimately inhibits DNA and RNA synthesis. Modified (Skubisz and Tong 2012)

3.4. Cyclophosphamide

Cyclophosphamide is an alkylating agent and is one of the most successful and widely utilized anticancer drugs. It was approved for clinical use as chemotherapeutic agent in 1959. It is commonly used for treatment of a wide range of hematological malignancies, solid tumors and autoimmune diseases such as systemic lupus erythematsous. Furthermore, it is commonly used in blood and marrow transplantation as an effective immunosuppressive agent (Emadi et al. 2009).

Cyclophosphamide is an inactive pro-drug and has to be activated by hepatic cytochrome P-450 enzymes, while its active form 4-hydroperoxy-cyclophosphamide does not require hepatic activation (Teicher et al. 1996). Although toxicity of cyclophosphamide is not cell-cycle specific, it is a cytotoxic agent for proliferating cells such as granulosa cells which are particular targets of action of cyclophosphamide (Plowchalk and Mattison 1992).

The mechanism behind cyclophosphamide-induced follicle loss is activation of primordial follicle by up-regulation of the phosphatidylinositol 3-kinase (PI3K/PTEN/Akt) signaling pathway and causing growing follicles to undergo apoptosis, in this manner reducing of secretion of inhibitory factors. At the end, cyclophosphamide causing burn out and depletion of the ovarian reservoir, because increase of activation primordial follicles which are recruited into growth, develop and die (Fig. 6). In absence this disturbance in balance caused by cyclophosphamide, the ovary is in a state of equilibrium during normal follicular growth and the primordial follicles are under regulation of PI3K/PTEN/Akt pathway with action of suppressive factors (Kalich-Philosoph et al. 2013).

Cyclophosphamide has extremely damaging effects on the ovary and carries highest risk of gonadal dysfunction (Meirow et al. 1999, Wallace et al. 2005). One single dose of cyclophosphamide caused loss of primordial follicles and follicles of all stage in ovaries of mice (Kalich-Philosoph et al. 2013). In another study on mice, cyclophosphamide treatment caused reduction in ovarian volume, destruction of antral follicle and low production of estradiol (Plowchalk and Mattison 1992). Similar results showed a reduction in ovarian weight in rats treated with cyclophosphamide (Sato et al. 2009).

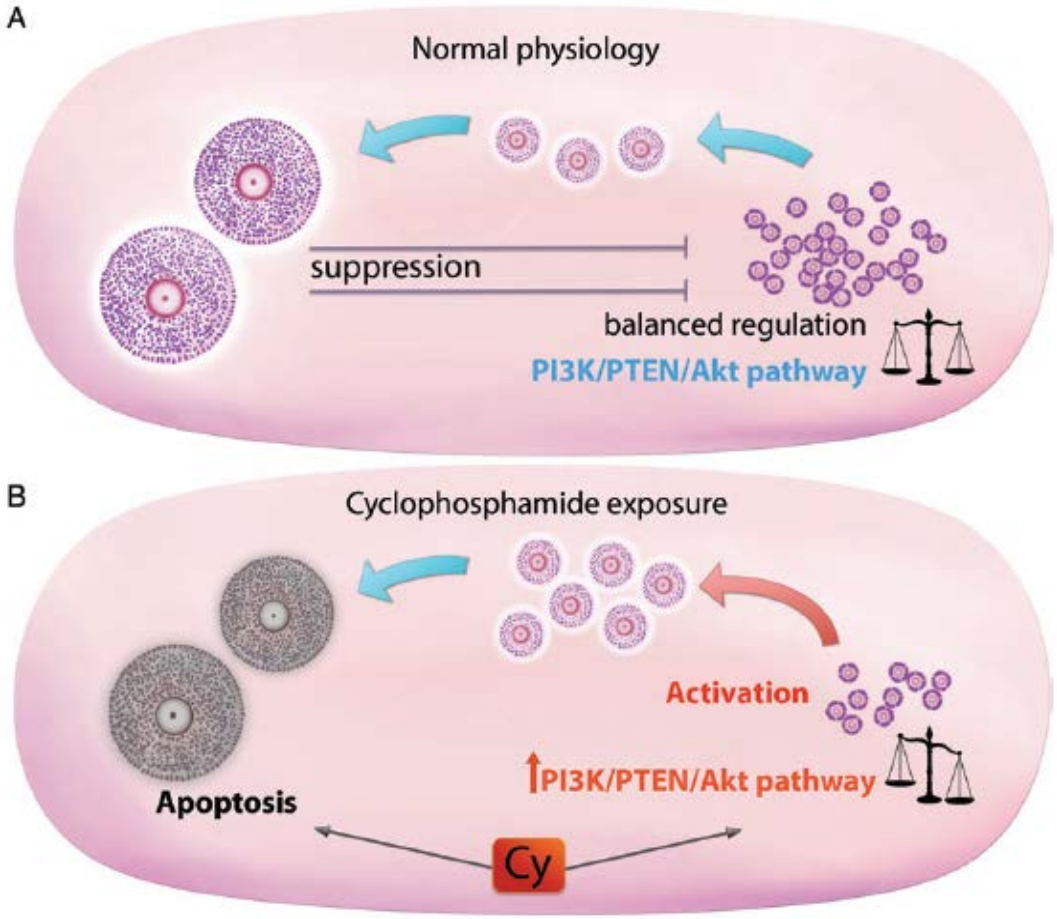


Figure 6. Induction of follicle loss by cyclophosphamide
(A) The PI3K/PTEN/Akt pathway and suppressive factors produced by growing follicles maintain a state of equilibrium during normal follicular development. (B) Treatment with cyclophosphamide causes imbalance and up-regulation of the PI3K/PTEN/Akt follicle activation pathway and causes apoptosis of growing follicles. At the end, burn out occurs in ovarian reservoir due to death of primordial follicles. Modified (Roness et al. 2014).

4. Chemotherapy and risk of infertility of female survivors of childhood cancer

After successful treatment, young female cancer survivors still have an elevated risk for late effects of chemotherapy treatments, such as a possible premature ovarian failure and infertility (Byrne et al. 1987, Byrne 1999, Sklar 2005, Green et al. 2009, Hamre et al. 2012, Thomas-Teinturier et al. 2013). A morphological study of the ovaries of leukemic children treated with cytotoxic agents demonstrated an inhibition in follicle development and a decrease of number and size of antral follicles in comparison to control group (Himelstein-Braw et al. 1978). A structural study of the ovary in childhood leukemia after chemotherapy treatment showed a reduction in the number of follicles, absence of follicles in two postmenarcheal girls, moderate to severe signs of fibrosis in cortical stroma and changes in capillaries (Marcello et al. 1990).

The Childhood Cancer Survivor Study followed a large number of patients diagnosed between 1970 and 1986 and found that female long-term survivors of childhood cancer were less likely to become pregnant compared to sibling controls (Green et al. 2009). Up to one third of adults who had been treated for cancer in childhood or in adolescence have suspected infertility in a large nationwide study of fertility in Germany (Balcerek et al. 2012). In a prospective cohort study on prepubertal and pubertal girls treated for cancer, AMH level was measured before, during and after each chemotherapy treatment. During chemotherapy, AMH was undetectable in 50% of patients and 100% of patients in the low/medium risk groups and high-risk group respectively. Hence, Anti-Müllerian Hormone is a useful and sensitive marker of ovarian reserve and gonadal function in women treated for cancer during childhood and can be detectable in girls of all ages (Brougham et al. 2012).

Chemotherapy treatment in childhood that disrupts fertility in adulthood can have a shattering effect, both at the time of treatment and whenever the patient's wishes to start family. Patients are in need to identify best options to minimize ovarian damage during chemotherapy. Therefore, the effect of chemotherapy is important to consider, as well as which strategies are available to protect fertility in later life.

5. Impact of chemotherapy on the ovary

The impact of chemotherapy on the ovary includes short and long-term effects depending on the severity of damage of primordial follicle population. During the treatment, damage of growing follicles leads to temporary amenorrhea which is an immediate effect of chemotherapy, while destruction of the primordial follicle pool leads to long-term effect. Permanent infertility can occur as result of the complete loss of primordial follicle reserve (Roness et al. 2014).

Chemotherapy induces reduction of the primordial pool by two possible ways: direct killing of primordial follicles or indirect by damage of growing follicles which leads to the recruitment of more primordial follicles, both ways leading to premature ovarian failure (Fig. 7 A) (Meirow et al. 2010). The various cell types in the ovary are potential targets of action of chemotherapeutic drugs. While non-dividing oocytes in the interior of the immature follicle may be a potential target for direct damage by cytostatic drugs via initiation of cell death and subsequent loss of germ cells, mitotically- active granulosa cells are the preferably targets of chemotherapeutic agents (Fig. 7 B). Direct damage of granulosa cells leads to indirect damage of oocyte and causes loss of germ cell (Morgan et al. 2012).

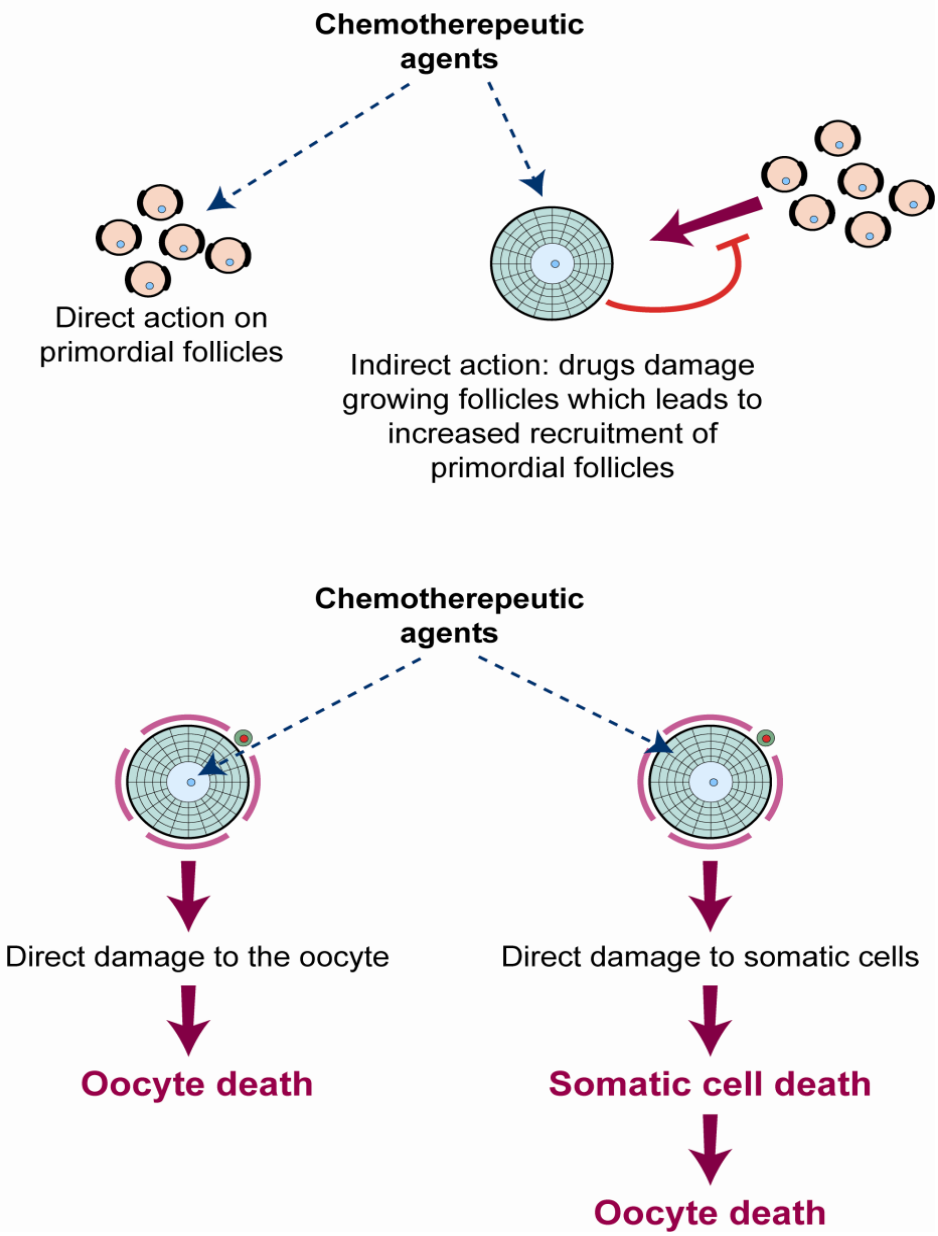


Figure 7. Several potential targets in the ovary for toxic actions of chemotherapy
(A) Direct damage of the primordial follicle pool or indirect damage of growing follicle damage caused by action of chemotherapeutics agents. More of primordial follicles are recruited into growth and so loss of that reserve due to loss inhibitory action of growing follicles. (B) Chemotherapy could directly damage the oocyte or somatic cells, which will lead indirect damage to oocyte and loss of follicle loss. Modified (Morgan et al. 2012).

6. Effect chemotherapy on granulosa cells

Granulosa cells are mitotically and metabolically active cells and proliferate during follicle maturation. Therefore, they are the primary ovarian targets to action of chemotherapeutics agents. Any damage of granulosa cells will lead to indirect damage of oocytes and hidden effects on primordial follicles (Morgan et al. 2012). This damage occurs mainly by inducing apoptosis in granulosa cells (Himelstein-Braw et al. 1978, Marcello et al. 1990, Meirow et al. 2010, Chatterjee et al. 2014, Yuksel et al. 2015).

In response to cytostatic drugs causing DNA damage, cells attempt to activate DNA repair mechanisms by a complex cascade response and failure of this attempt results in apoptosis. Apoptosis as mechanism of programmed cell death results in characteristic cytomorphological changes including cell shrinkage, chromatin condensation, membrane blebbing and formation apoptotic bodies (Elmore 2007). Apoptosis of granulosa cells in growing follicles is mediated by a Fas antigen- specific death inducer called FasL transmembrane protein (Perez et al. 1999, Utsunomiya et al. 2008)

In a previous study, treatment with doxorubicin caused apoptosis of granulosa cells and oocytes of human primordial follicles (Soleimani et al. 2011). Similar results by another study identified DNA damage in granulosa cells when ovaries from mice were treated with doxorubicin (Ben-Aharon et al. 2010). An increase in damaged granulosa cell nuclei was observed in vitro when human ovarian cortical slices from premenopausal women were treated with cyclophosphamide (Raz et al. 2002). Other previous studies investigated effects of cyclophosphamide and doxorubicin on granulosa cells (Table 1).

Summarized, granulosa cell apoptosis is one of the most important factors affecting the oocyte quality and has been associated with oocyte maturation delay and consequently deficient embryo development and poor pregnancy outcome (Lee et al. 2001, Host et al. 2002).

Table 1. Previous studies on effects of cyclophosphamide and doxorubicin on granulosa cells

Chemotherapy regimen	Author	Model species	Target cells
Cyclophosphamide	(Ataya et al. 1990)	Human Rat	Granulosa cells
	(Tsai-Turton et al. 2007)	Human granulosa cell lines	COV434
	(Zhao et al. 2010)	Rat	Granulosa cells
	(Bildik et al. 2015)	Human Human granulosa cell lines	Granulosa cells COV434, HGrCl and HLGC
	(Yuksel et al. 2015)	Human Human granulosa cell lines Rat granulosa cell lines	Granulosa cells COV434, HGrCl and SIGC
Doxorubicin	(Soleimani et al. 2011)	Human	Granulosa cells
	(Sanchez et al. 2013)	Human	Granulosa cells
	(Turgeman et al. 2014)	Human	Granulosa cells

7. Chemotherapy and Premature Ovarian Failure

Premature Ovarian Failure means development of amenorrhoea due to premature depletion of functional ovarian follicle which leads to amenorrhea before age 40 (Goswami und Conway 2005). The efficacy of chemotherapy on ovarian function depends on patient's age, chemotherapeutic protocol and dose of drug administered (Meirow et al. 2010, Ben-Aharon und Shalgi 2012).

The chemotherapeutic agents can be categorized according to their ovarian failure risk into three groups: high, medium and low risk (Wallace et al. 2005, Blumenfeld 2012). Alkylating agents possess the highest risk in causing ovarian failure. (Meirow 2000) found that risk of ovarian failure in patients treated with alkylating agents was 4.52 fold higher than in patients who were not treated with these drugs, while clinical studies found no risk of ovarian failure in patients treated with Vinca alkaloids (Meirow 2000, Lee et al. 2006).

Moreover, treatment with doxorubicin and cyclophosphamide caused amenorrhea in 84% of treated women, but about half of them recovered the menstrual bleeding during nine months after treatment (Petrek et al. 2006). A cohort study of childhood cancer survivors who received chemotherapy including alkylating agents showed that, of the six female survivors, one developed premature ovarian insufficiency (Larsen et al. 2003).

Risk of developing premature ovarian failure and infertility following chemotherapy has associated risks including cardiovascular disease and psychosocial problems, such as depression (Carter et al. 2005, Jeanes et al. 2007).

The end result of the chemotherapy-induced damage to dividing cells of the ovary, granulosa cells, as well as to the oocyte, is premature ovarian failure leading to premature menopause and permanent infertility.

8. MicroRNAs: small molecules with important functions in reproduction

Recently, a large family of short, noncoding RNA molecules known as microRNAs (miRNAs) has begun to be explored in ovarian cells because of their critical regulatory role (Imbar und Eisenberg 2014). miRNAs have been identified more than 20 years ago as key posttranscriptional regulator of gene expression (Lee et al. 1993). They are approximately 21 nucleotides long and represent approximately 1–3% of genes in humans and may regulate up to 90% of all genes (Silveri et al. 2006, Stefani and Slack 2006). miRNAs have a critical role in regulation of reproductive functions, especially in steroid synthesis, folliculogenesis, oocyte maturation, implantation and early embryonic development, differentiation and apoptosis (Sirotkin et al. 2009, Hossain et al. 2009, Donadeu et al. 2012). The authors suggested that miRNAs can control reproductive functions by enhanced or inhibited release of ovarian progestagen, androgen and estrogen in human granulosa cells and suggested that such miRNA-mediated effects may be potentially used for the regulation of reproductive processes including fertility and for the treatment of reproductive and other steroid dependent disorders of women (Sirotkin et al. 2009, Sang et al. 2013, Diez-Fraile et al. 2014).

Nearly 2865 mature miRNAs have been identified in the human genome until now (Virant-Klun et al. 2016) and the miRNA database miRBase is rapidly expanding with hundreds of new miRNA sequences added with every update. Therefore, in recent years, miRNAs have become a major focus of research in molecular biology. The first study on miRNA profiles in human mature oocytes and surrounding cumulus granulsoa cells identified three miRNAs abundant in oocytes (miR-184, miR-100 and miR-10A). They differed from the miRNAs abundant in cumulus cells in the ovarian follicle (Assou et al. 2013). Other studies analysed the miRNA profile in human ovarian granulosa cells (Sirotkin et al. 2009, Sirotkin et al. 2010, Tong et al. 2014, Xu et al. 2015) in human follicular fluid and mural granuloa cells (Moreno et al. 2015), in human granulosa cells tumor cell lines KGN and COV434 (Rosario et al. 2013) and in ovarian cells of different species like mouse (Fiedler et al. 2008, Ahn et al. 2010), cow (Hossain et al. 2009), pig (Zhou et al. 2015), sheep (McBride et al. 2012) and horse (Schauer et al. 2013). The stability of miRNAs in body fluids is a property that enables them to be used as novel biomarkers for detection of several human physiologic and pathologic processes, as described for follicular fluid of women with PCOS (Sang et al. 2013, Xu et al. 2015, Jiang et al. 2015).

8.1. Importance of miR-21 and miR-132 in granulosa cells

miR-21 and miR-132 are among the most abundantly expressed miRNAs in human oocytes and granulosa cells (Assou et al. 2013, Velthut-Meikas et al. 2013). In human follicular fluid (Sang et al. 2013) and in mouse granulosa cells, they have been demonstrated to be upregulated after LH and human chorionic gonadotropin induction (Fiedler et al. 2008). Because miR-21 is an oncogenic miRNA playing a critical role in maintaining the survival of granulosa cells in periovulatory follicles and acting as an antiapoptotic factor in granulosa cells (Donadeu et al. 2012), it is a particularly interesting target of study.

In miR-21-depleted mice apoptosis is significantly increased in ovaries in vivo in combination with transient increase in phosphatase and tensin homolog protein (PTEN) levels in granulosa cells (Carter et al. 2005, Fiedler et al. 2008, Hennebold 2010). In several studies, miR-132 has been demonstrated to be upregulated in rat granulosa cells by either cAMP (Hu et al. 2013) or FSH treatment. Xu et al. (2015) identified the role of miR-132 in estradiol synthesis in mouse granulosa cells (Xu et al. 2015). Their mimics increased estradiol secretion and their inhibitors decreased estradiol secretion. The mechanisms by which miR-132 regulates estradiol secretion remain unknown and are worthy of investigation in the future (Sang et al. 2013).

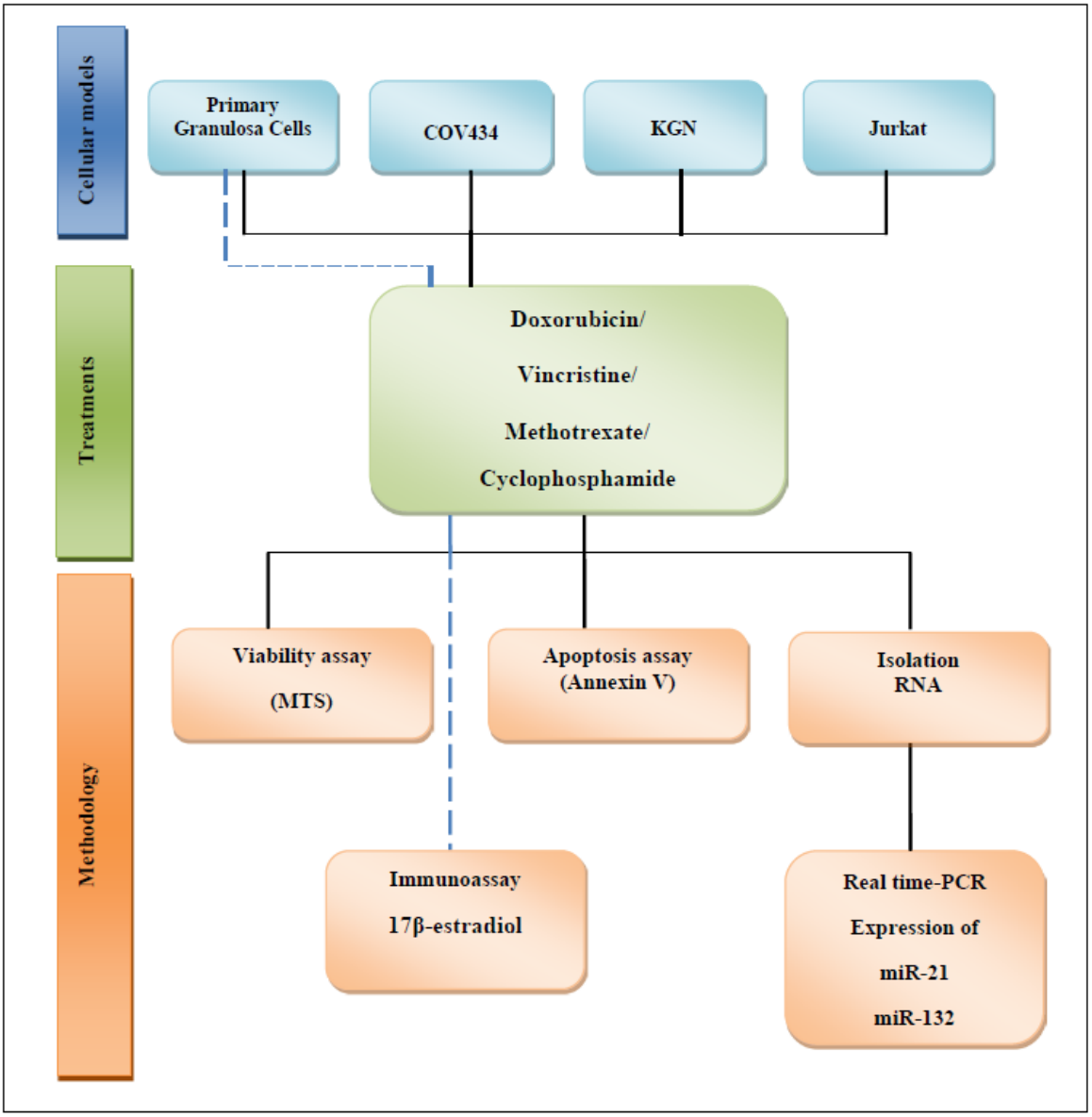
CHAPTER 2 OBJECTIVE

- To determine the potential association of different chemotherapeutic agents as part of childhood cancer treatment with infertility in adults who survived pediatric cancer.

- To compare the effects of doxorubicin, vincristine, methotrexate and cyclophosphamide on a human leukemia T cell line, granulosa cell lines and primary granulosa cells.

- To explore effects of chemotherapeutic agents on expression of miR-21 and miR-132 in a primary granulosa cells, granulosa cell lines and a human leukemia T cell line.

2.1 Experimental Design



CHAPTER 3 MATERIALS AND METHODS

3.1. Chemicals and reagents

Doxorubicin, vincristine, and methotrexate were gifts from the Pharmacy of the University Hospital Jena. All drugs were diluted in medium to obtain the different concentrations. Chemotherapy drugs were administered at their therapeutic blood concentrations.

3.1.1. Active metabolite of cyclophosphamide: 4-hydroperoxy-cyclophosphamide

Our preliminary experiments with 1 to 500µg/ml cyclophosphamide for 12-48 h did not increase apoptosis in cell lines as referred by non-significant decrease in cell viability at the highest dose, suggesting that these cells do not efficiently metabolize cyclophosphamide to its active metabolites. Therefore, the active metabolite of cyclophosphamide (4-hydroperoxy-cyclophosphamide) was used for the subsequent experiments. The active metabolite of cyclophosphamide was a kind gift from Baxter Oncology (Halle/Westfalen, Germany) as powder of 20 mg and it has been divided to aliquots and stored at -20°C.

3.2. Ethical aspects

Informed consent was obtained from each couple for the use of the follicular fluid sample that was obtained during oocyte retrieval for the in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment. This study was covered by a previous approval of local ethics committee at the Friedrich-Schiller University Jena.

3.3. Cell culture

Two cell lines, COV434, derived from a human granulosa cell tumor (Zhang et al.2000), and KGN, derived from steroidogenic human ovarian granulosa-like tumor cells (Nishi et al 2001), were cultured in cell culture medium Dulbecco's modified Eagle's medium (DMEM)/Ham's F12; (Life Technologies,Inc., Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Steinheim, Germany), and 1% penicillin/streptomycin (Life Technologies, Grand Island, New York, USA). The human leukemia T cell line Jurkat was maintained in RPMI 1640 (Life Technologies) and supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown under standard conditions (37°C, 5% CO_2, humidified tissue culture incubator).

Furthermore, mycoplasma contamination of all cell lines was excluded by using mycoplasma kits (INtRON biotechnology Inc. Sungnam, Korea) every six months.

Table 2. Characterization and origin of cell lines

Cell line	Characterization	Culture properties	Source	Reference
COV434	Immortalized human granulosa cells (primary tumor)	Adherent	Health Protection Agency Culture Collection via Sigma-Aldrich	(Zhang et al. 2000)
KGN	Granulosa tumor cell	Adherent	Dr. Jana Ernstat the Halle University Hospital, Germany	(Nishi et al. 2001)
Jurkat	Immortalized human leukemia T cell	Suspension	Dr. John Hansen at the Fred Hutchinson Cancer Research Center, Seattle, USA	(Abraham und Weiss 2004)

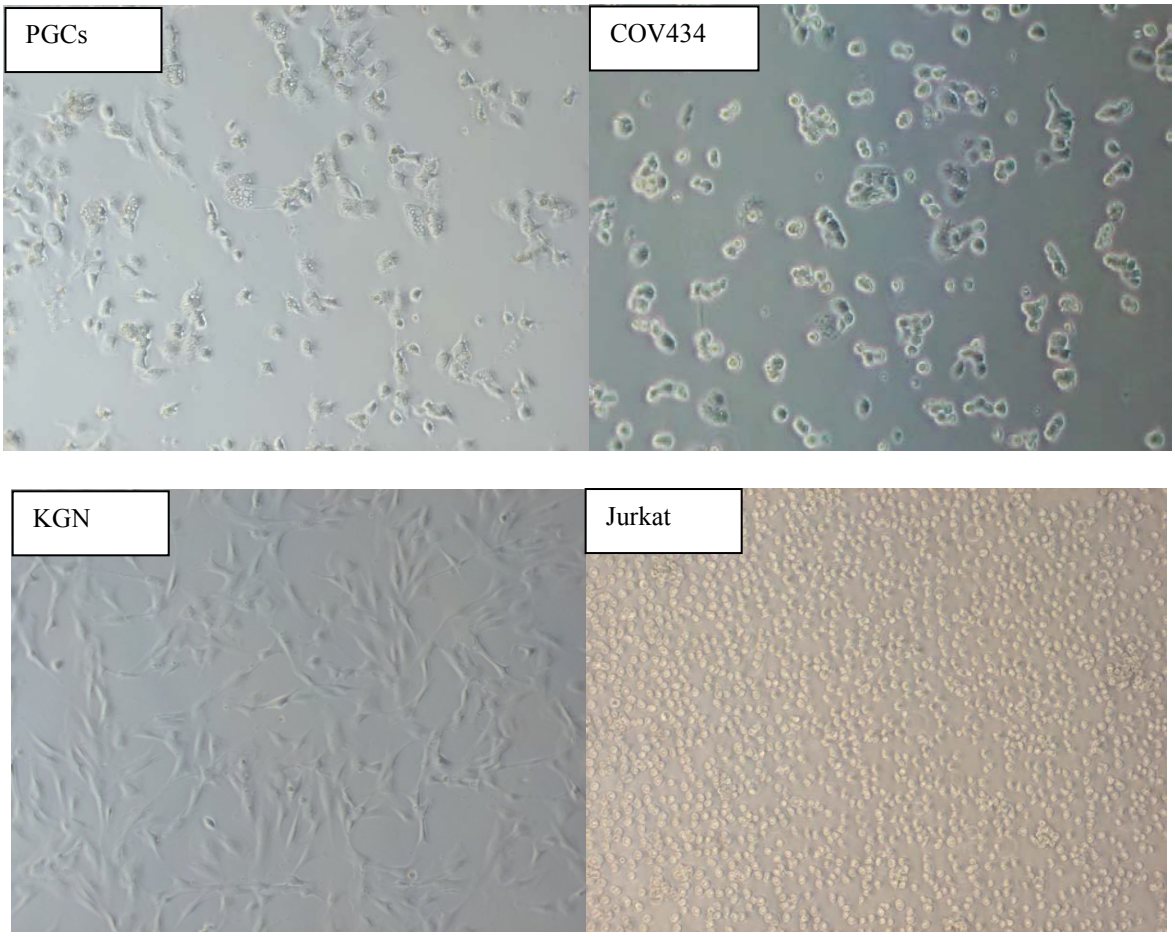


Figure 8. Human primary cells and cell lines used for this thesis
Human primary granulosa cells (PGCs). Human granulosa cell lines COV434 and KGN (adherent cell lines). Human leukemia T cell line Jurkat (suspension cell line).

3.4. Isolation of human primary granulosa cells and culture

Ovarian follicular fluids (FF) were obtained from women undergoing infertility treatment with (IVF) or (ICSI) at the Reproductive Medicine Center in Jena. There was no information about the patients, stimulation protocol, medical treatments, or number of aspirated follicles, because of data protection. Importantly, FF from all aspirated follicles of each patient was pooled. In some cases, FF from two patients or the isolated granulosa cells from several patients were pooled to obtain sufficient numbers of granulosa cells. The protocol for isolation was established in previous studies in our Placenta Laboratory. In brief, primary granulosa cells were collected from the FF aspirates and were centrifuged initially for 5 min at 700 × g, followed by layering them onto a 33% Percoll gradient (GE Healthcare Life Science), followed by a centrifugation for 20 min at 400 × g. Thereafter, 3 layers can be distinguished: a top layer containing the follicular fluid, a bottom layer containing erythrocytes, and a middle ring-like layer containing granulosa cells. This middle layer was collected, washed, resuspended in DMEM (Life Technologies) with 10% FBS(Sigma Aldrich) and 0.05% gentamycin (Life Technologies) and cultured at 37°C and 5% CO_2 for 48 hours to recover from effects of the in vivo exposure to gonadotropins. Cell suspensions were washed daily with phosphatase buffered saline (PBS) and medium was changed to remove the contaminating leukocytes, that do not adhere to the culture flask, whereas granulosa cells adhere, thus allowing easy segregation of the two cell types (Ferrero et al. 2012).

3.5. Cell viability assay (MTS)

Primary granulosa cells were cultured for 72 h and seeded in a 96-well cell culture plate at a density of 2×10^4 cells/well in triplicates. After further 24 h, the media was changed and different doses of doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 μg/ml), vincristine (0.01, 0.1, 1, 5, and 10 μg/ml), methotrexate (0.1, 1, 5, 10, and 50 μg/ml) orcyclophosphamide (1, 5,10, 50 and 100 μg/ml) were added and cells were incubated for further 48 h. The granulosa cell lines (KGN and COV434) and the leukemia T cell line Jurkat were treated for 12, 24 and 48 h with the same doses of doxorubicin, vincristine, methotrexate or cyclophosphamide. The cells incubated with medium only served as a control. Subsequently, cell viability was determined using a colorimetric methyl tetra-zolium salt (MTS)3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H tetrazolium) Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, USA). 20 μl of MTS solution were added to each well, followed by incubation for 4 h. Absorbance was read at 490 nm on a Spectro star Omega microtiter plate reader. The wells containing medium but no cells served as blank controls.

Viability of test samples was determined as: % viability= (average OD for test group/average OD for control group) ×100. The values were obtained from at least three independent experiments (three wells each).

3.6. Apoptosis (Annexin V assay)

Apoptosis assessment was measured using a FITC conjugated annexin V and Propidium Iodide (PI) apoptosis kit (Miltenyi Biotechnology, Germany) by flow cytometry. According to the manufacturers' instructions**,** cells were seeded in 6 well plates at a density of $5x10^5$ cells/well with 2 ml growth medium for 24 h to reach adherence and confluence, followed by incubation of cells with the first concentration which inhibited cell viability > 50% of either doxorubicin, vincristine, methotrexate or cyclophosphamidefor further 48 h. Adherent and floating cells were collected and then washed and resuspended in 1x binding buffer, followed by adding 100µl of buffer and 10µl of annexine V-FITC solution (which binds extracellularly to apoptotic cells) and cells were incubated in dark at room temperature for 15 min. Cells were washed with binding buffer and suspended in 500 µl of binding buffer and 5µl of Propidium iodide (which stains necrotic cells) were added to cell suspension and followed by flow cytometer (BD FACSCalibur) and data analyzed by CellQuest Pro Program (BecktonDickinson, Mountain View, California). Apoptotic cells were Annexin V-FITC positive and necrotic cells were PI positive.

3.7. RNA Isolation

Primary granulosa cells, KGN, COV434 and Jurkat were seeded in 6-well plates at a density of 5×10^5 cells/well for 24 h, followed by treatment with 1µg/ml doxorubicin or vincristine or methotrexate or cyclophosphamide for further 24 h. Total cellular RNA was extracted from cultured cells using TRIZOL reagent according to the manufacturer´s instructions (Invitrogen, Darmstadt, Germany). Total RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (PeqLab Biotechnologies GmbH, Erlangen, Germany). Samples with purity ratio more than 1.8 at A260/A280 were stored at -80°C until being processed.

3.8. Reverse transcription

cDNA (complementary deoxyribonucleic acid) was synthesised using the Taqman microRNA reverse transcription kit (Applied Biosystems,Darmstadt, Germany) according to manufacturer's protocol. In brief, reverse transcription reactions were set up in a reaction volume of 15 µl by combining 7 ml of reverse transcription (RT) master mix, including deoxynucleotide triphosphates (dNTPs), RT buffer, MultiScribeRT and RNase inhibitor. 5 ml of 10 ng of total RNA was combined with 3 ml of reverse transcription primer specific for each miRNA. Reverse transcription reaction was performed using a thermocycler (Eppendorf, Germany).

Table 3. Reverse transcription reaction program

Step	Temperature in°C.	Time in minutes
1	16	30
2	42	30
3	85	5
4	4	-

3.9. Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Taqman microRNA assay for (hsa-miR-21-5p, Assay ID: 000397; hsa-miR-132-3p, Assay ID: 000457; RNU-43 Assay ID: 001006) and Taqman universal PCR master mix reagents (Applied Biosystems) according to manufacturer's protocol. Briefly, 10 ml of TaqMan 2x Universal PCR master mix was combined with 7.67 ml of nuclease-free water, 1 ml of 20x TaqMan MicroRNA. All reactions were run in duplicates, including no-template controls in 96-well plates on a 7300 Real-time PCRsystem (Applied Biosystems). Experiments were performed in triplicates. Expression of miR-21 and miR-132 was calculated using the $2^{-\Delta\Delta Ct}$method by relative to the housekeeping small nucleolar RNA U48 (RNU48). $\Delta\Delta Ct = (Ct_{miR\text{-}21/\ miR\text{-}132} - Ct_{RNU48})$ treated group$- (Ct_{miR\text{-}21/\ miR\text{-}132} - Ct_{RNU48})$ control group.

Table 4. Real-time PCR program

Step	Temperature °C.	Time	Number of Cycles
Activation	95	10 min	1
Denaturation	95	15s	40
Annealing/ Extension	60	60s	

3.10. Hormone assay

The level of estradiol was determined in supernatants of primary granulosa cell cultures after 48 h incubation of cells with different doses of either doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), vincristine (0.01, 0.1, 1, 5, and 10 µg/ml),methotrexate (0.1, 1, 5, 10, and 50 µg/ml) or cyclophosphamide (1, 5,10, 50 and 100 µg/ml). The analyses have been done by using an immunoassay for the in vitro quantitative determination of estradiol (immunoassay analyzers, Roche Diagnostics, USA) by the Institute of Clinical Chemistry, University Hospital Jena. The lower detection limit for estradiol was 18.4 pmol/l. The experiments were repeated at least three times.

3.11. Statistics

Each experiment was repeated independently at least three times. Two-tailed Student's t-test was performed for statistical comparisons between experimental groups and values were presented as mean ± standard deviation (SD). Statistical analyses for miR-21 and miR-132 expression levels were assessed by ANOVA with Turkey multiple-comparison test (GraphPad Prism version 6.0). P values <0.05 were considered statistically significant.

CHAPTER 4 RESULTS

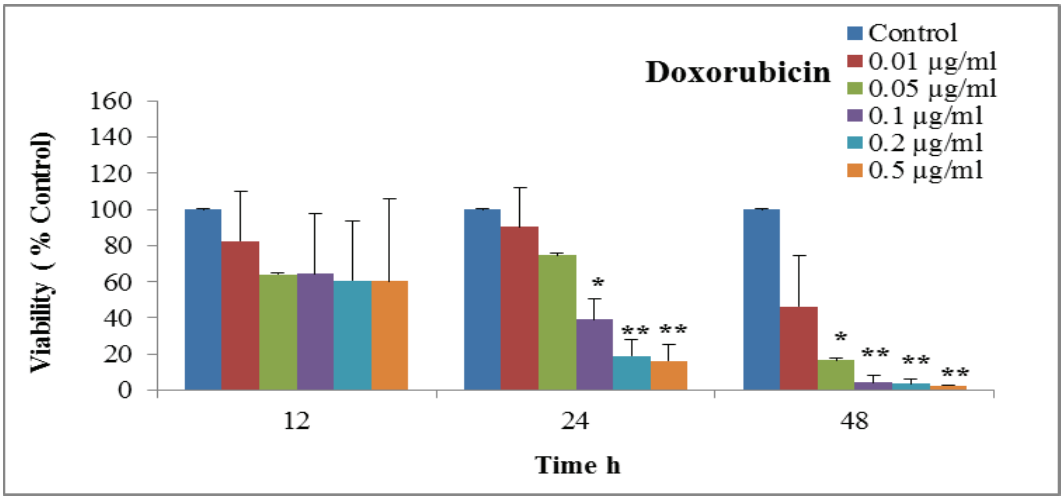
4. 1. Effect on cell viability

Cells were incubated with escalating doses of doxorubicin, vincristine, methotrexate or cyclophosphamide for 12, 24 and 48 h. The functional investigation shows the effect of cytostatic drugs on cell viability (n=3).

4.1.1. Effect chemotherapeutic drugs on viability in Jurkat cells

The viability of the human leukemia T cell line Jurkat upon cytostatic drugs treatment decreased in dose- and time-dependent manner with respect to controls. After 12 h, there was significant reduction of viability in cells, that were treated with vincristine and cyclophosphamide at increasing concentrations (1, 5, 10 µg/ml and 10, 50, 100 µg/ml) (Fig.1B and D). After 48 h, reduction of viability was higher than in cells treated for 24 h (Fig. 9A-D).

A



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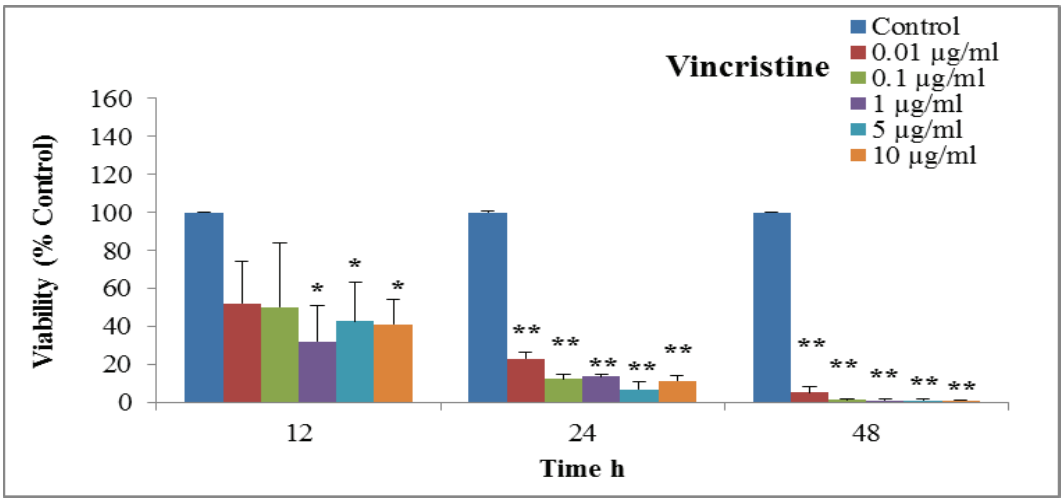

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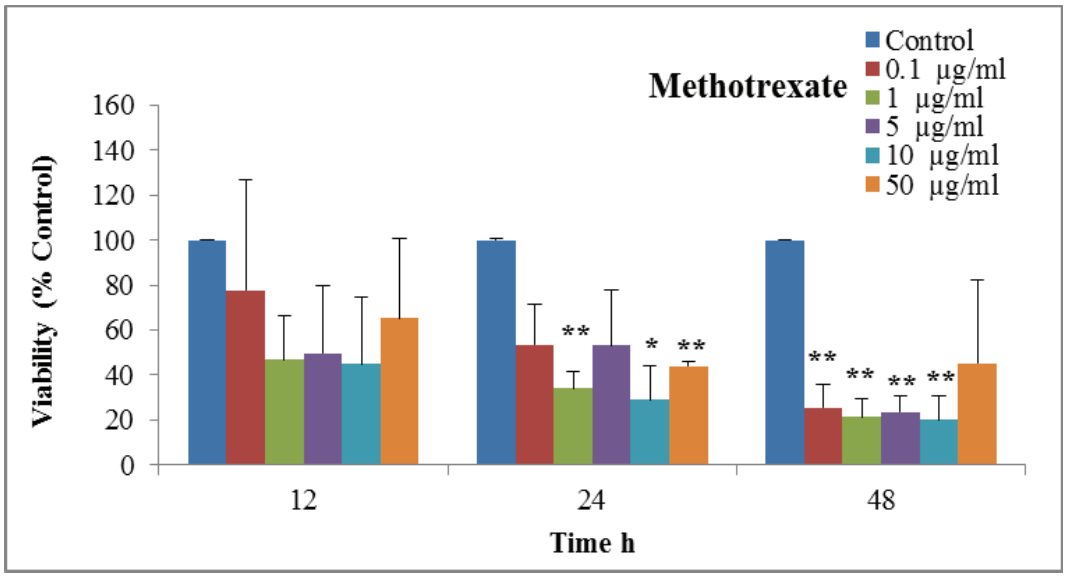

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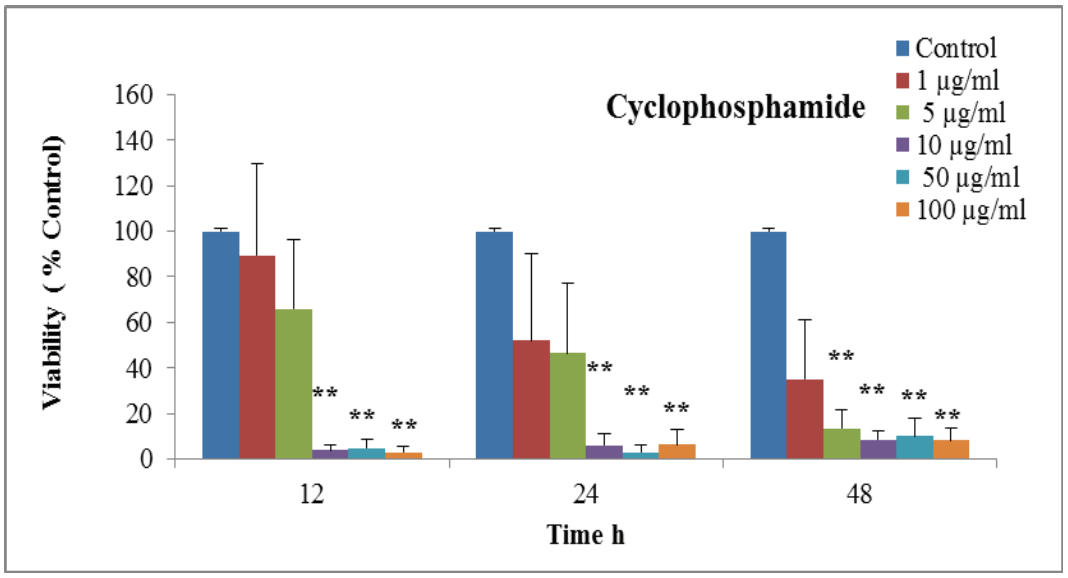
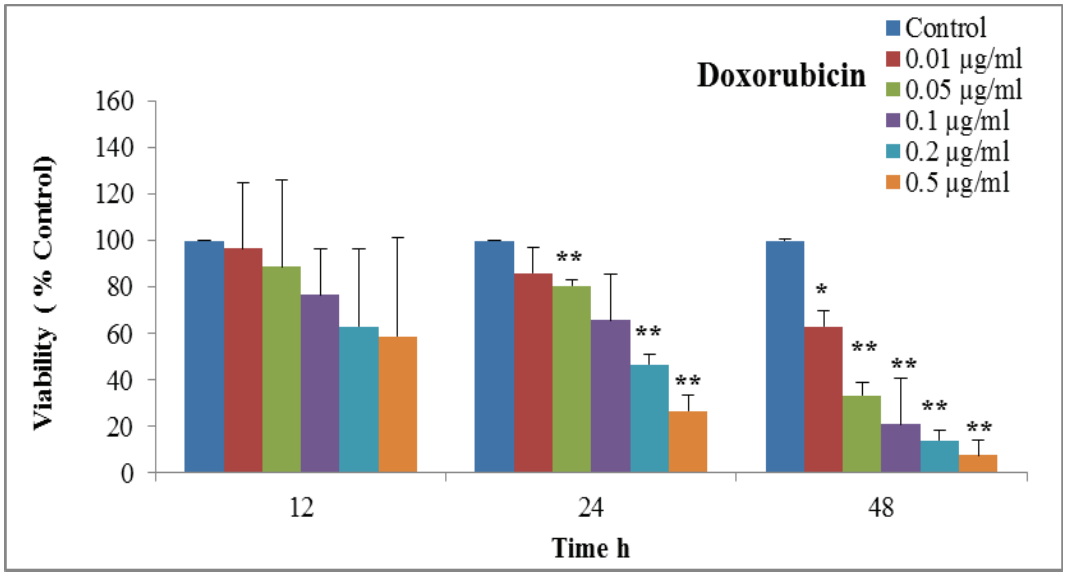


Figure 9. Effects of chemotherapeutic drugs on viability in Jurkat cells.
Cells were untreated, treated with increasing doses of (A) doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), (B) vincristine (0.01, 0.1, 1, 5, and 10 µg/ml), (C) methotrexate (0.1, 1, 5, 10, and 50 µg/ml) or (D) cyclophosphamide (1, 5,10, 50 and 100 µg/ml) for 12 h, 24 h or 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, *$p < 0.05$, **$p < 0.01$(Student's t test).

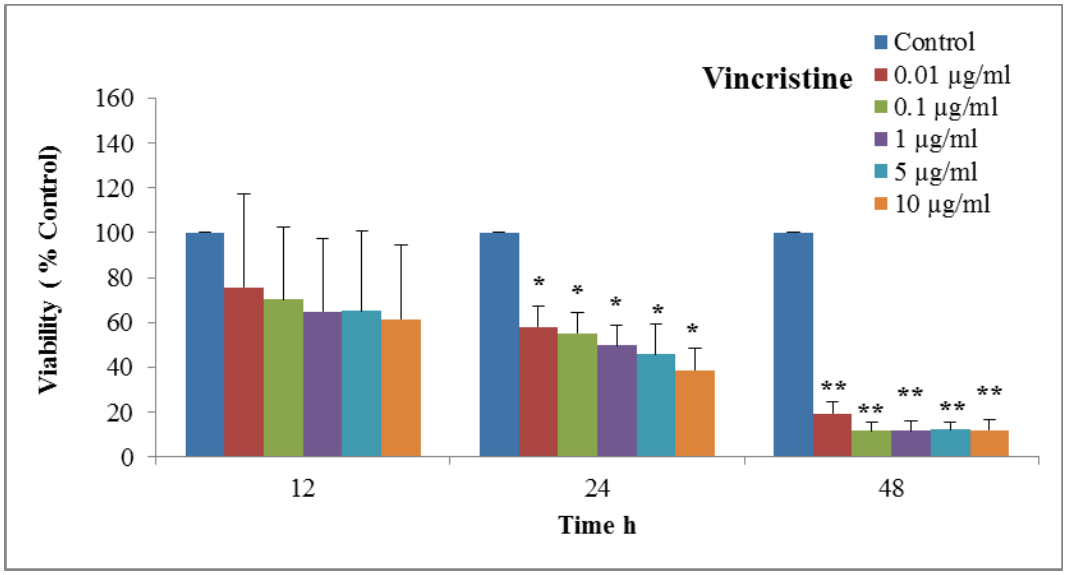
4.1.2. Effect chemotherapeutic drugs on viability in COV434 cells

In COV434 cells, there was significant decrease in viability after 12 h at increasing concentrations (10, 50 and 100 µg/ml) of cyclophosphamide (Fig. 10 D). Methotrexate had no inhibitory effect on viability in COV434 cells (Fig. 10 C), although after 48 h, there was a significant decrease in viability at all concentrations of the other tested drugs (Fig. 10A, B, D).

A



B



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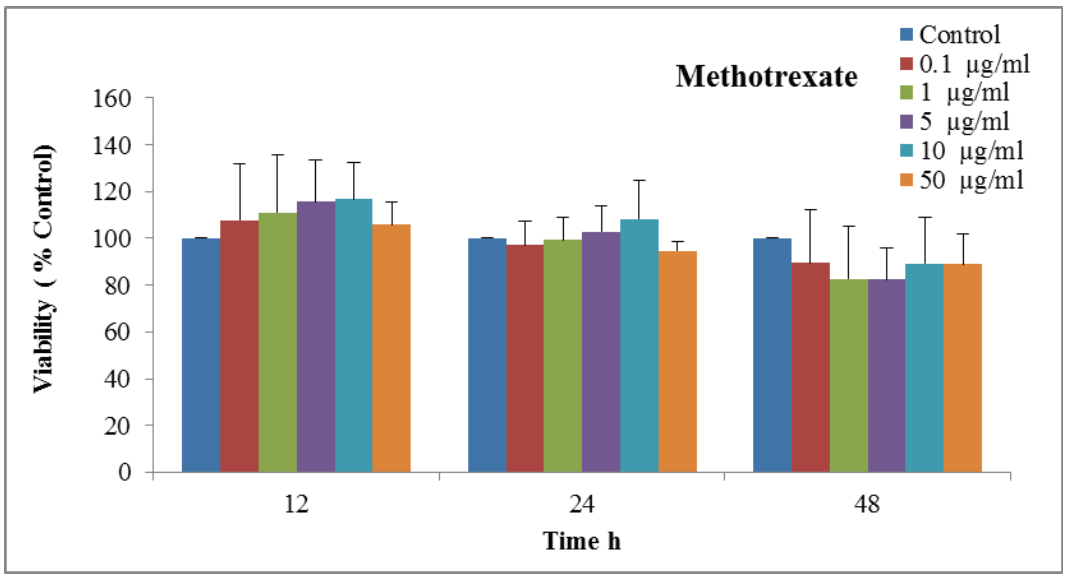

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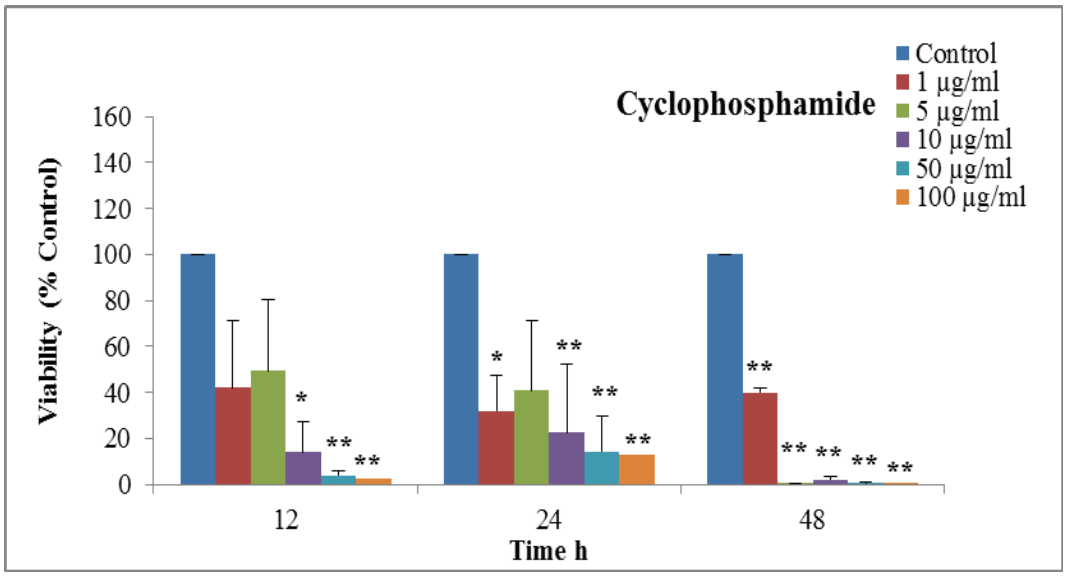
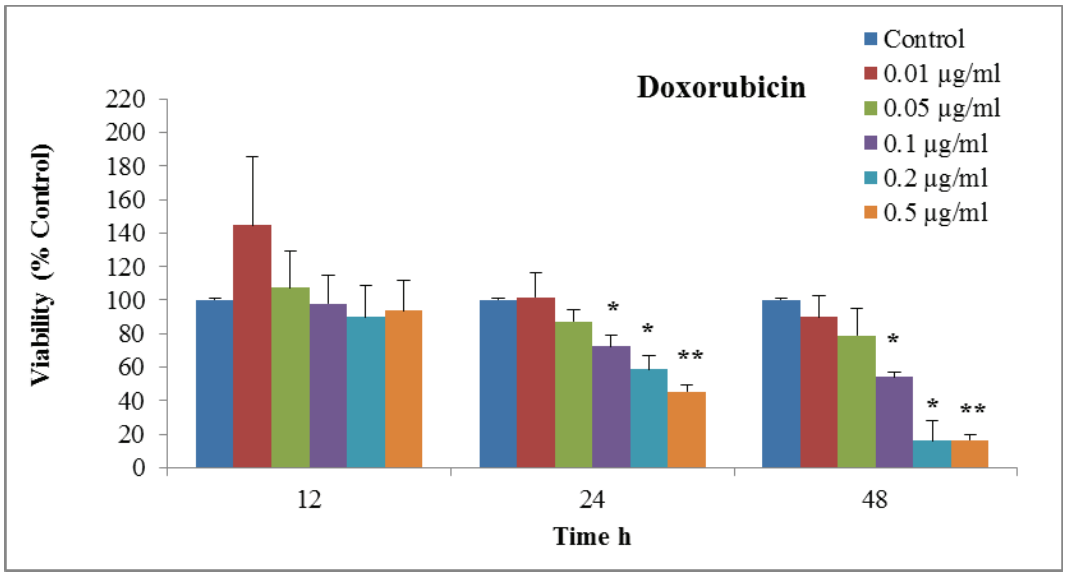


Figure 10. **Effects of chemotherapeutic drugs on viability in COV434 cells.**
Cells were untreated, treated with increasing doses of (A) doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), (B) vincristine (0.01, 0.1, 1, 5, and 10 µg/ml), (C) methotrexate (0.1, 1, 5, 10, and 50 µg/ml) or (D) cyclophosphamide (1, 5,10, 50 and 100 µg/ml) for 12 h, 24 h or 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments performed in triplicates, *$p < 0.05$, **$p < 0.01$(Student's t test).

4.1.3. Effect chemotherapeutic drugs on viability in KGN cells

The viability of KGN after treatment with doxorubicin and vincristine decreased in dose- and time-dependent manner in comparison to controls (Fig. 11A and 3B), whereas, treatment with methotrexate had no cytotoxic effect on cells (Fig. 11C). Remarkably, there were no viable cells at 50 and 100 µg/ml of cyclophosphamide in all time points (Fig. 11D).

A



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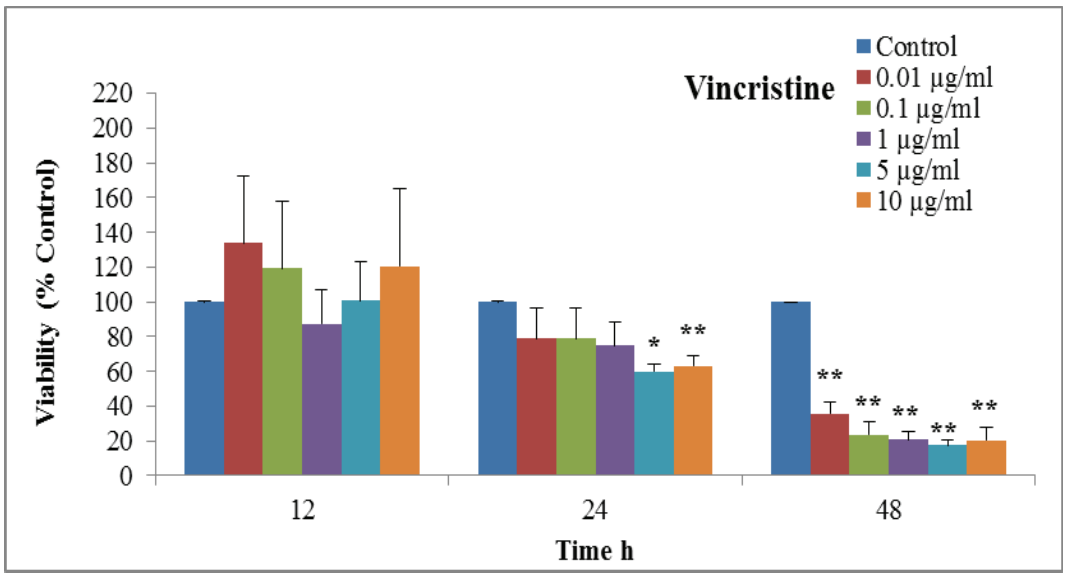

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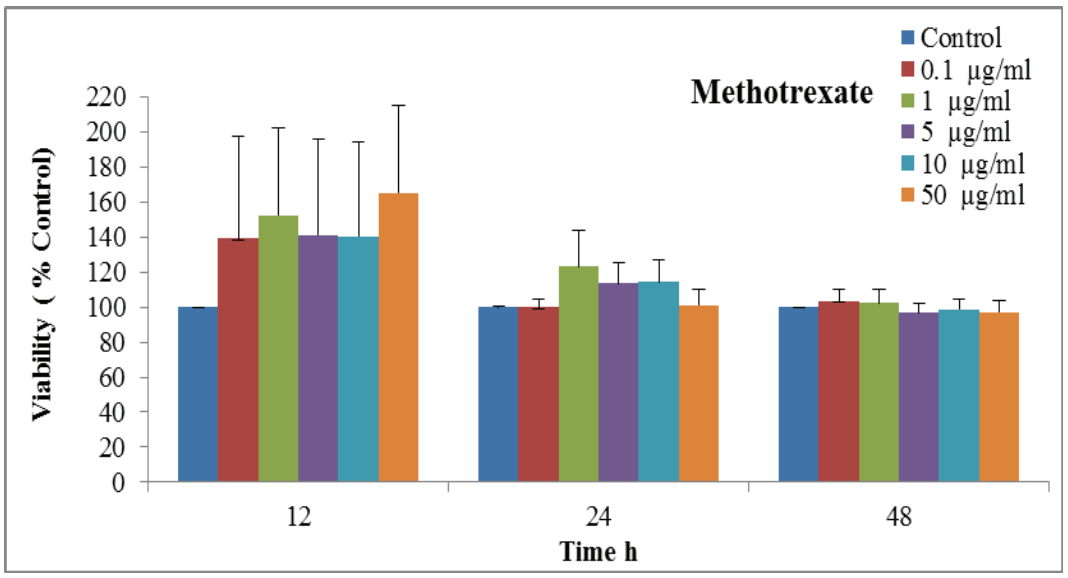

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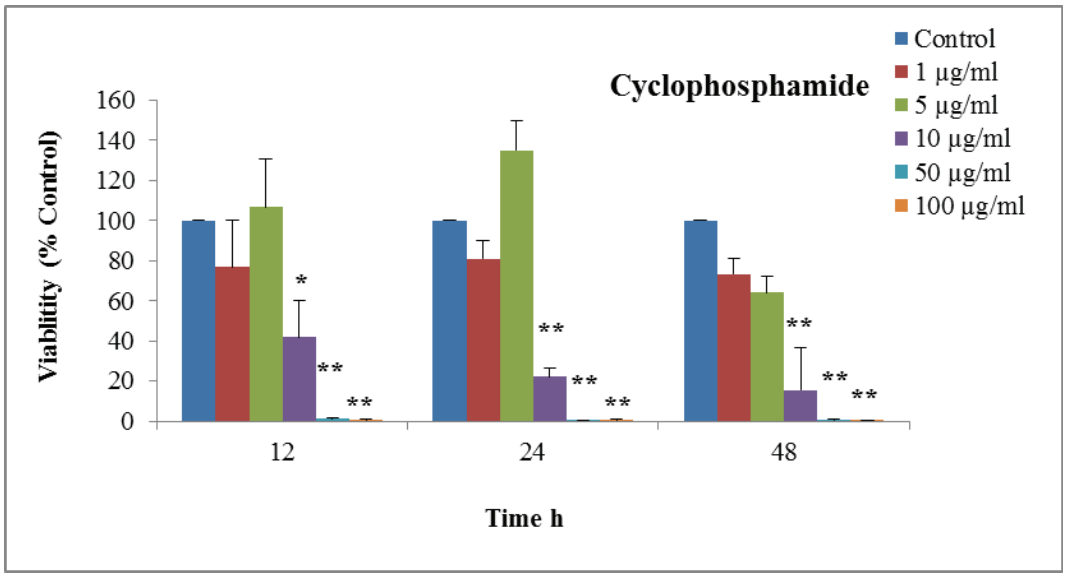


Figure 11. **Effects of chemotherapeutic drugs on viability in KGN cells**.
Cells were untreated, treated with increasing doses of (A) doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), (B) vincristine (0.01, 0.1, 1, 5, and 10 µg/ml), (C) methotrexate (0.1, 1, 5, 10, and 50 µg/ml) or (D) cyclophosphamide (1, 5,10, 50 and 100 µg/ml) for 12 h, 24 h or 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, *$p < 0.05$, **$p < 0.01$ (Student's t test).

4.1.4. Effect chemotherapeutic drugs on viability in human primary granulosa cells

Cells were incubated with increasing doses of doxorubicin, vincristine, methotrexate or cyclophosphamide for 48 h, because the most toxic effects of all drugs were observed after 48 h treatment. Doxorubicin and cyclophosphamide induced a significant reduction in cell viability at (0.5 µg/ml) and (50 and 100 µg/ml), respectively, whereas treatment with vincristine and methotrexate had no significant cytotoxic effects on cells (Fig. 12).

A

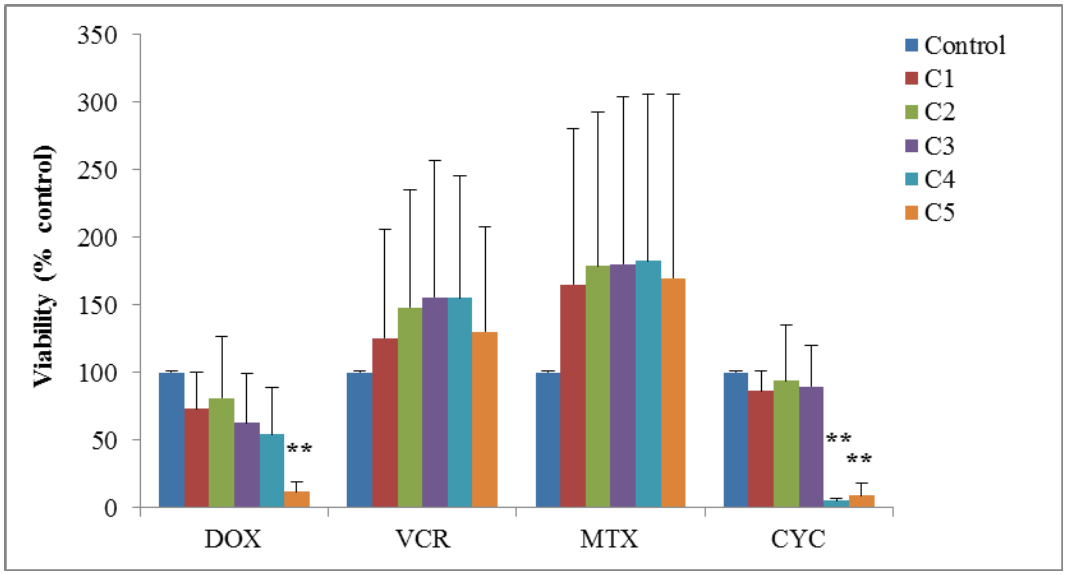


Figure 12. **Effects of chemotherapeutic drugs on viability in primary granulosa cells**.
Cells were untreated and treated with increasing doses (C1 to C5) of doxorubicin (DOX; 0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), vincristine (VCR; 0.01, 0.1, 1, 5, and 10 µg/ml), methotrexate (MTX; 0.1, 1, 5, 10, and 50 µg/ml) or cyclophosphamide (CYC; 1, 5, 10, 50 and 100 µg/ml) for 48h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments each performed in triplicate, **p < 0.01 (Student's t test).

4.2 Effects of chemotherapeutic drugs on steroidogenesis in primary granulosa cells

Assessment of functionality of primary granulosa cells was carried out by measurement of secreted estradiol upon incubation with cytostatic drugs in a dose- and time-dependent manner. The results showed that primary granulosa cells produced detectable amounts of E2 in vitro. Treatment of primary granulosa cells with cyclophosphamide (1-100 µg/ml) for 48 h resulted in a dramatic decline in hormone level. Similar cytotoxic effects were observed in granulosa cells exposed to varying doses of doxorubicin (0.01-0.5 µg/ml). Treatment of cells with vincristine and methotrexate did not cause a notable change in their steroidogenic activity (Fig. 13). The above data are in line with the effects of cytostatic drugs on cell viability.

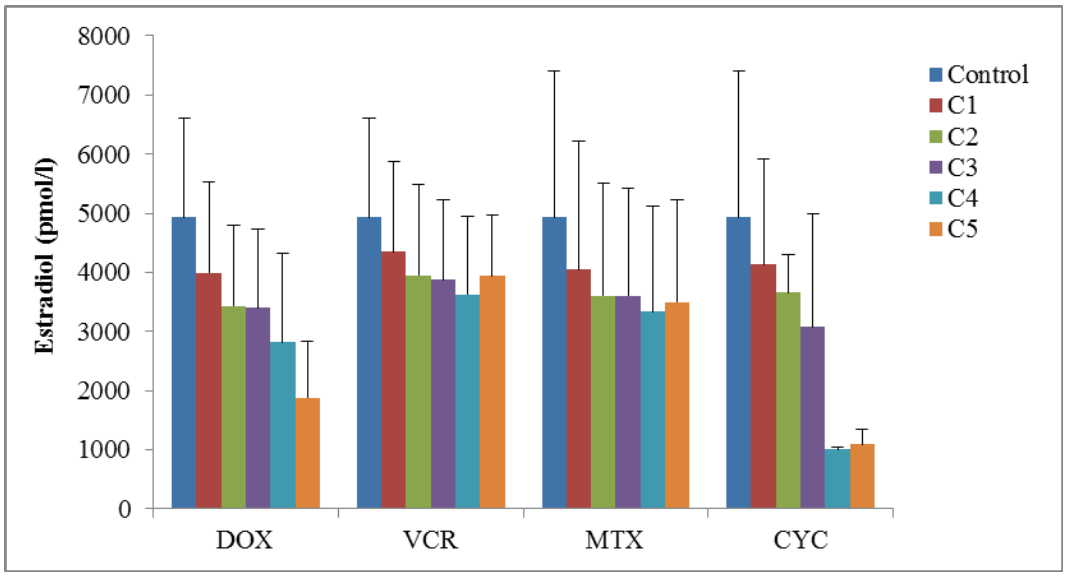


Figure 13. **Effects of chemotherapeutic drugs on secretion of estradiol in primary granulosa cells** Cells were untreated, treated with increasing doses of doxorubicin (DOX; 0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml), vincristine (VCR; 0.01, 0.1, 1, 5, and 10 µg/ml), methotrexate (MTX; 0.1, 1, 5, 10, and 50 µg/ml) or cyclophosphamide (CYC; 1, 5, 10, 50 and 100 µg/ml) for 48 h. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, **$p < 0.01$ (Student's t test).

4.3 Comparisons of effects of drugs on viability of cells

In comparison to primary granulosa cells and granulosa cell lines, Jurkat cells exhibited the highest sensitivity to all tested substances in a dose- and time-dependent manner (Fig. 14-17). A strong reduction of cell viability was also induced by doxorubicin, vincristine and cyclophosphamide in both granulosa cell lines COV434 and KGN (Fig. 14, 15 and 17). Doxorubicin and cyclophosphamide also decreased viability of isolated granulosa cells (Fig. 14 and 17). Treatment with methotrexate induced cell death in Jurkat cells, while the magnitude of this response in KGN, COV434 and primary granulosa cells was significantly less pronounced $p<0.05$ (Fig. 16).

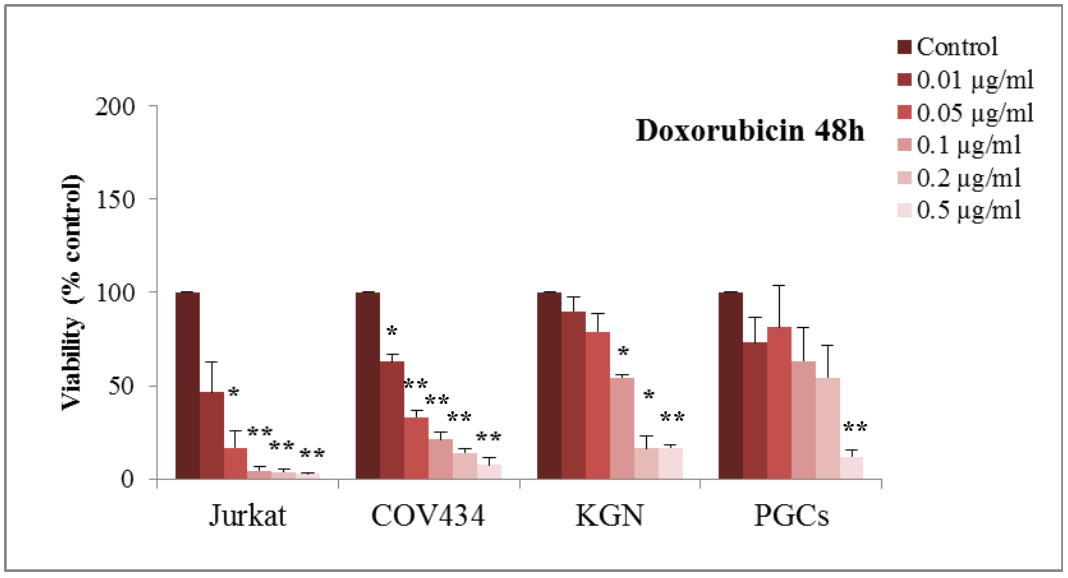


Figure 14. Effects of doxorubicin on viability in different cell lines.
Jurkat, COV434, KGN and primary granulosa cells (PGC) were untreated, treated with increasing doses of doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml) for 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, *$p < 0.05$, **$p < 0.01$ (Student's t test).

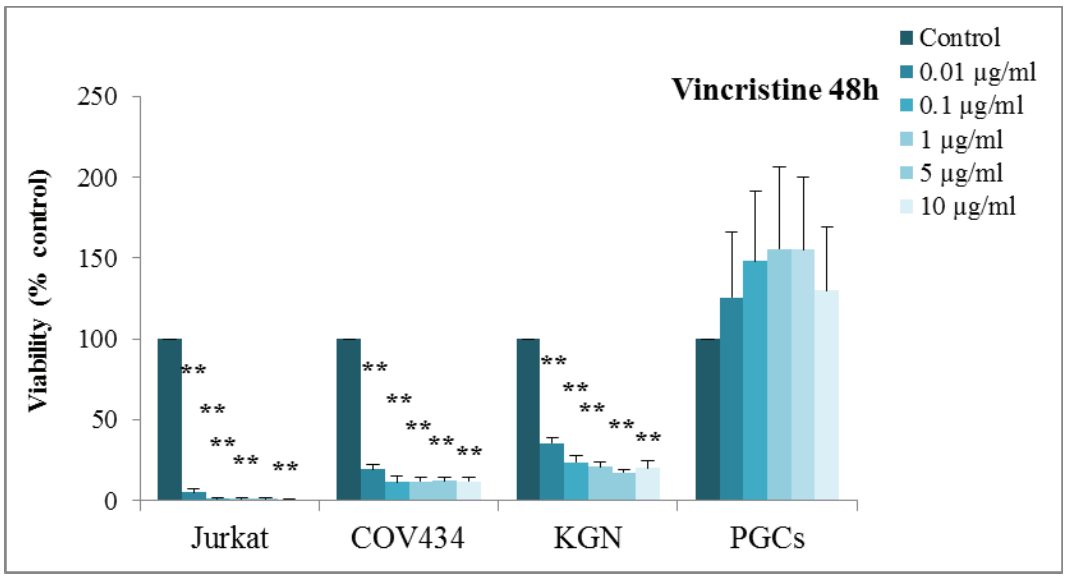


Figure 15. Effects of vincristine on viability in different cell lines.
Jurkat, COV434, KGN and primary granulosa cells (PGC) were untreated, treated with increasing doses of vincristine (0.01, 0.1, 1, 5, and 10 µg/ml), for 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, **p < 0.01 (Student's t test).

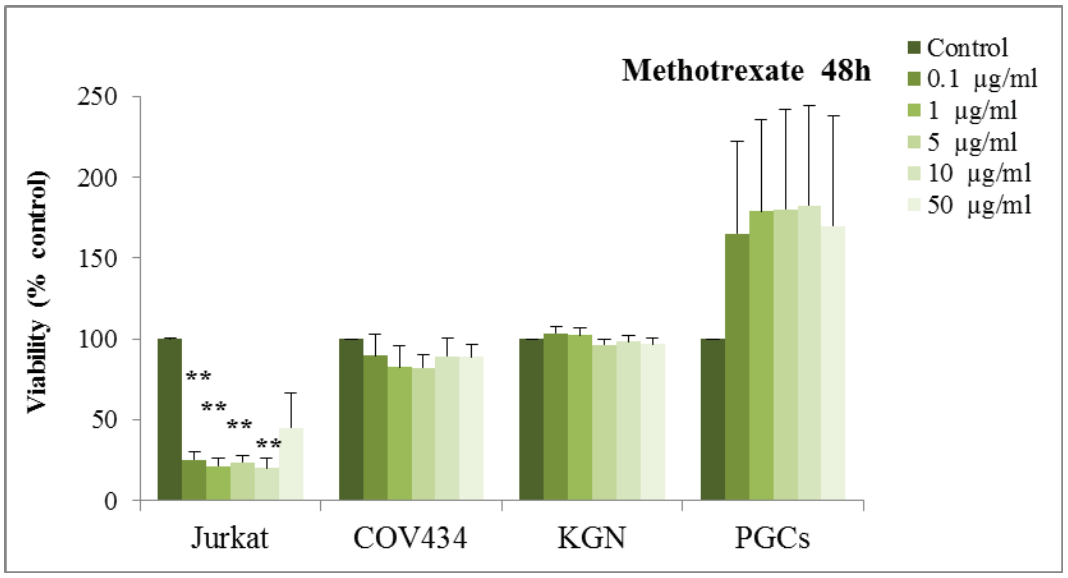


Figure 16. Effects of methotrexate on cell viability in different cell lines.
Jurkat, COV434, KGN and primary granulosa cells (PGC) were untreated, treated with increasing doses of methotrexate (0.1, 1, 5, 10, and 50 µg/ml) for 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, **p < 0.01 (Student's t test).

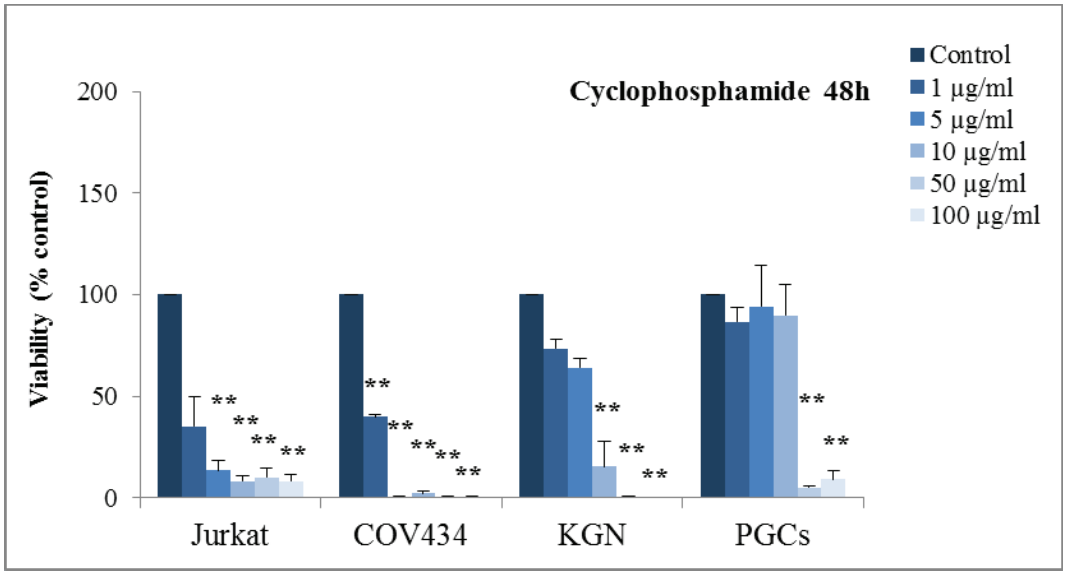


Figure 17. Effects of cyclophosphamide on cell viability in different cell lines.
Jurkat, COV434, KGN and primary granulosa cells were untreated, treated with increasing doses of cyclophosphamide (1, 5, 10, 50 and 100 µg/ml) for 48 h. Cell viability was determined by MTS assay. Results are presented as the mean±SD of 3 independent experiments, each performed in triplicates, **$p < 0.01$ (Student's t test).

4.4. Chemotherapeutic drugs promote apoptosis in cells

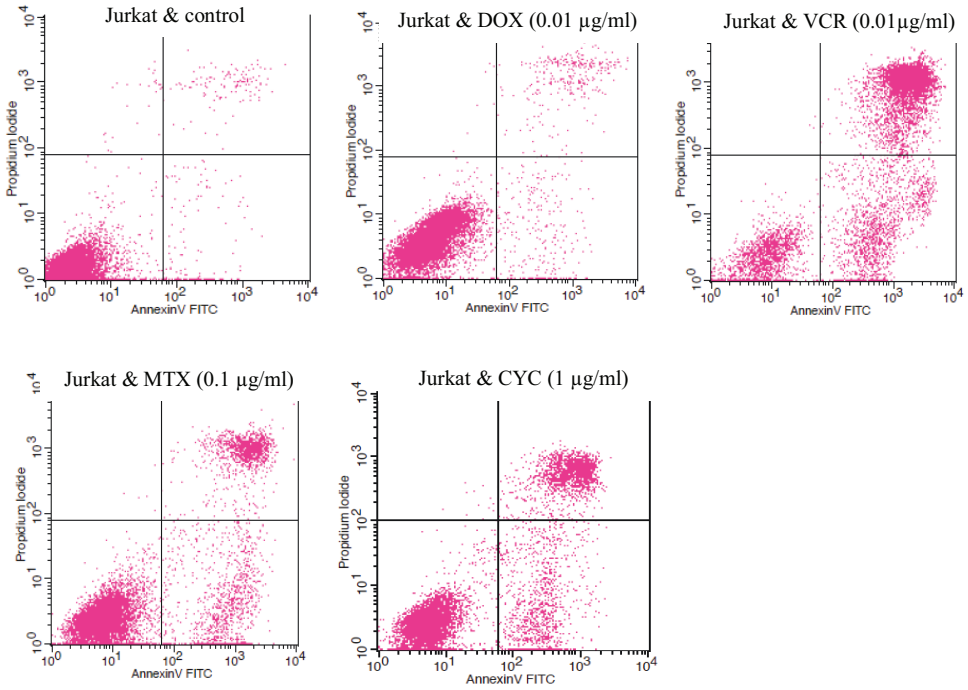
To assess the cytotoxic effects of chemotherapeutic drugs on apoptosis and necrosis of the different analyzed cells, cells were untreated, treated with first concentration which inhibited cell viability > 50% of either doxorubicin, vincristine, methotrexate or cyclophosphamide for 48 h and stained with annexin V and propodium iodide (PI) for flow cytometry.

In Jurkat cells, in response to vincristine the extent of necrosis (47.24%) was much higher than of apoptosis (21.62%). The lowest concentration was < 0.01 µg/ml of vincristine, applied for 48 h (Fig. 18 A and B). The percentage of apoptotic and necrotic KGN cells treated with the same concentration was below 10% each (Fig. 20 A and B).

The greatest magnitude of cytotoxicity appeared in primary granulosa cells treated with 50µg/ml cyclophosphamide and 0.5 µg/ml doxorubicin. Both induced apoptosis in approximately 80% of cells (Fig 21A and B). In untreated primary granulosa cells, 32,27±6,04% of cells were annexin V-positive/PI negative, and 14,71±3,8 % of cells were annexinV–PI double positive, because granulosa cells are non-mitotic cells which do not survive long time in culture.

Treatment with methotrexate induced significantly higher rates of cell death in Jurkat cells in comparison to control cells (Fig. 18 A and B) while it had low effect in COV434 cells, (Fig. 19 A and B), KGN cells (Fig. 20 A and B) and primary granulosa cells (Fig. 21 A and B). Doxorubicin induced apoptosis in KGN, while no evidence of apoptosis was observed after incubation of KGN cells with the other tested cytostatic drugs (Fig. 20).

A



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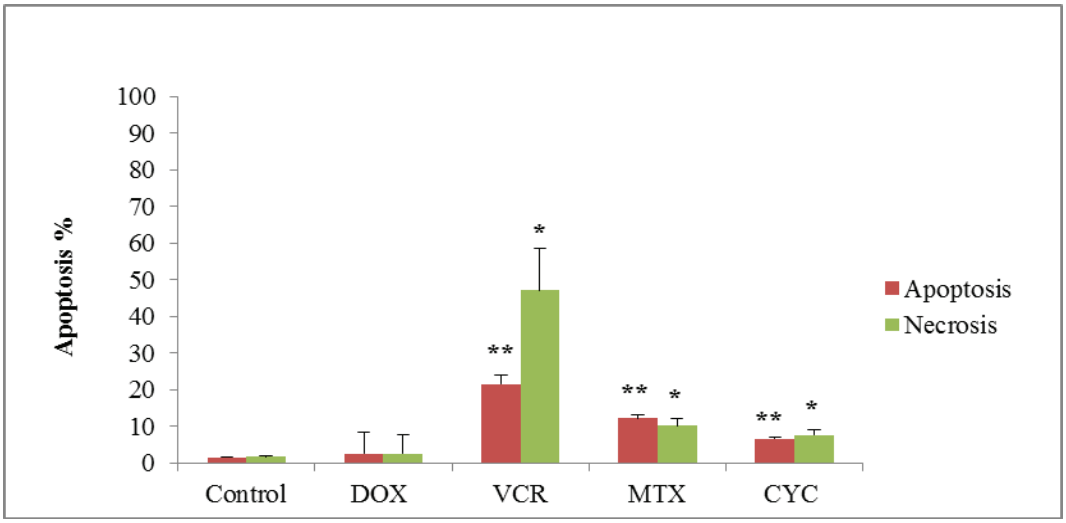
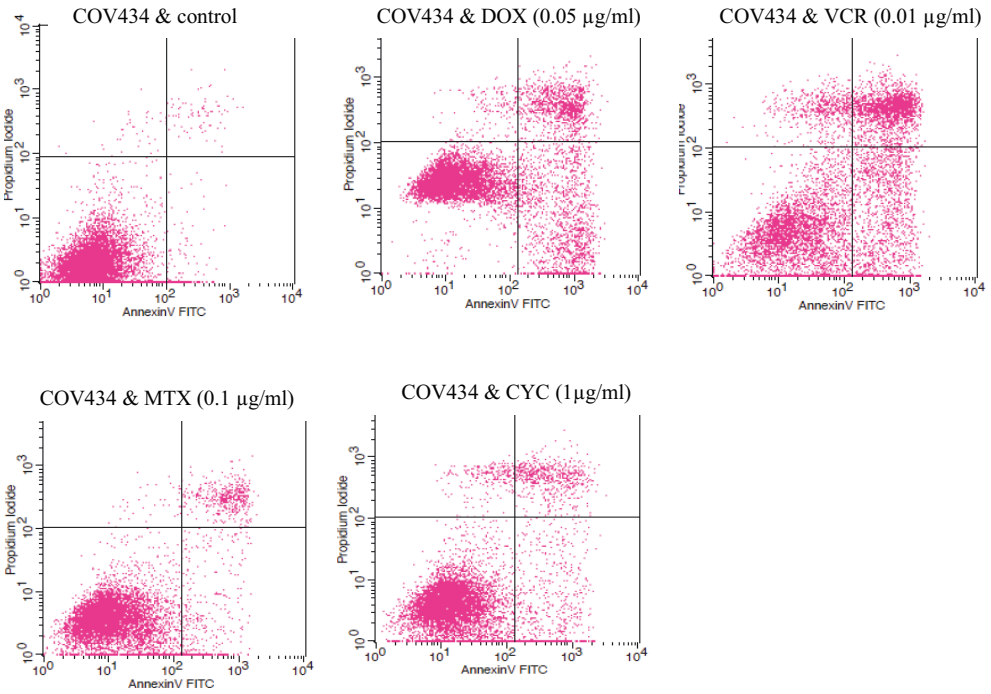


Figure 18. Apoptosis assay by annexin V showing cell death in Jurkat cells
(A) Flow cytometry for apoptosis assay by annexin V showing cell death in Jurkat cells untreated, treated with first concentration which inhibited cell viability > 50% of either doxorubicin (DOX; 0.01µg/ml), vincristine (VCR; 0.01 µg/ml), methotrexate (MTX; 0.1 µg/ml) or cyclophosphamide (CYC; 1 µg/ml) for 48 h and stained with annexin V and Propidium Iodide (PI). (B) The relative percentage of apoptosis and necrosis in Jurkat cells (n=3 independent experiments, Mean ± SD *p < 0.05, **p < 0.01; Student's t test).

A



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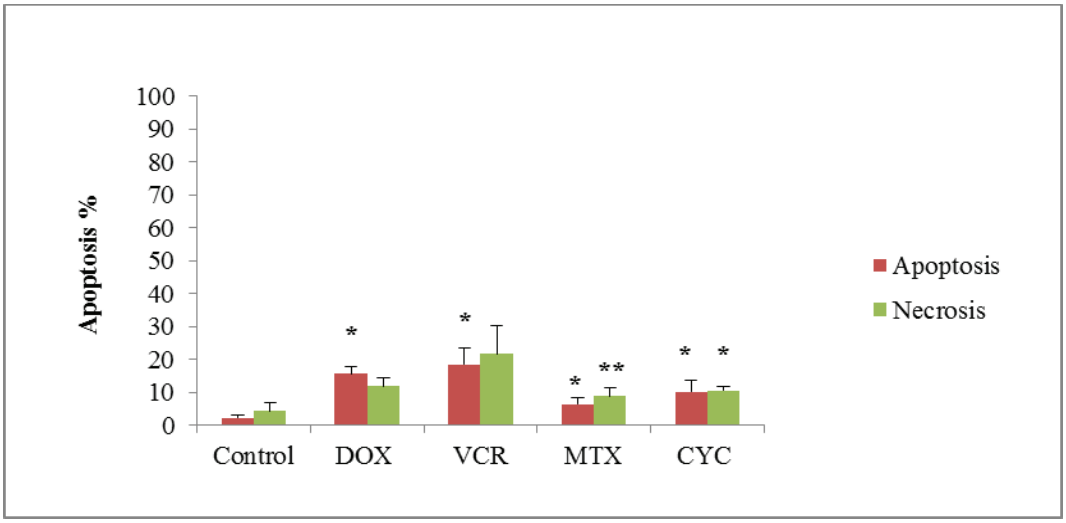
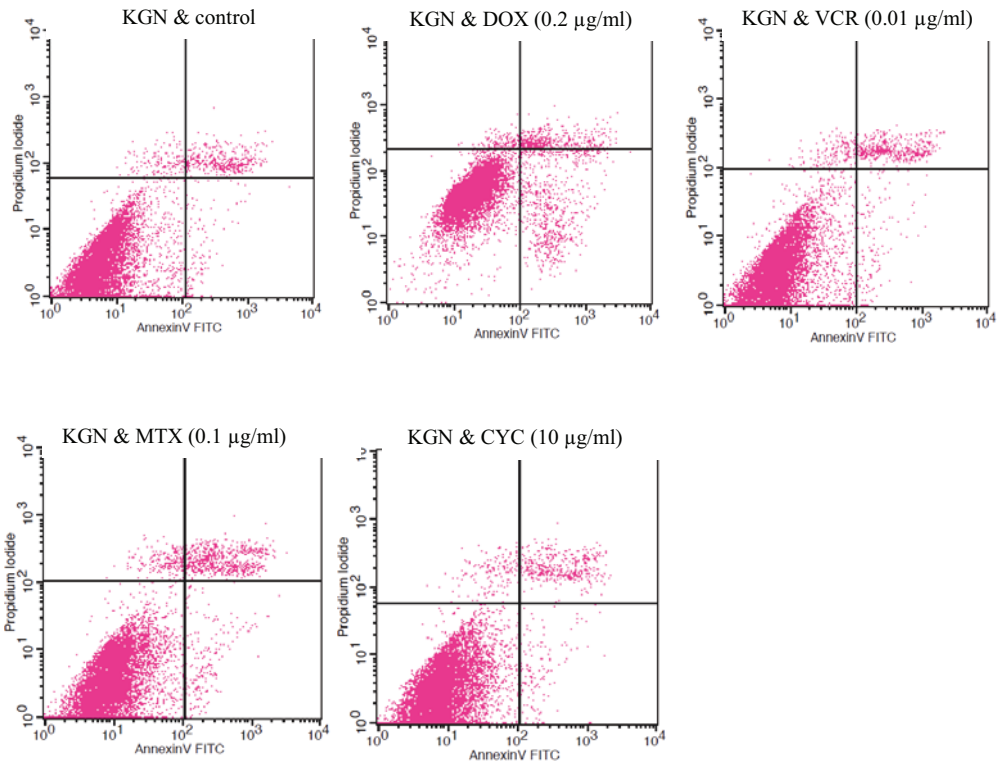


Figure 19. Apoptosis assay by annexin V showing the cell death in COV434 cells
(A) Flow cytometry for apoptosis assay by annexin V showing the cell death in COV434 cells untreated, treated with first concentration which inhibited cell viability > 50% of either doxorubicin (DOX; 0.05µg/ml), vincristine (VCR; 0.01 µg/ml), methotrexate (MTX; 0.1 µg/ml) or cyclophosphamide (CYC; 1 µg/ml) for 48 h and stained with annexin V and Propidium Iodide (PI). (B) The relative percentage of apoptosis and necrosis in COV434 cells (n=3 independent experiments, Mean ± SD *p < 0.05, **p < 0.01; Student's t test).

A



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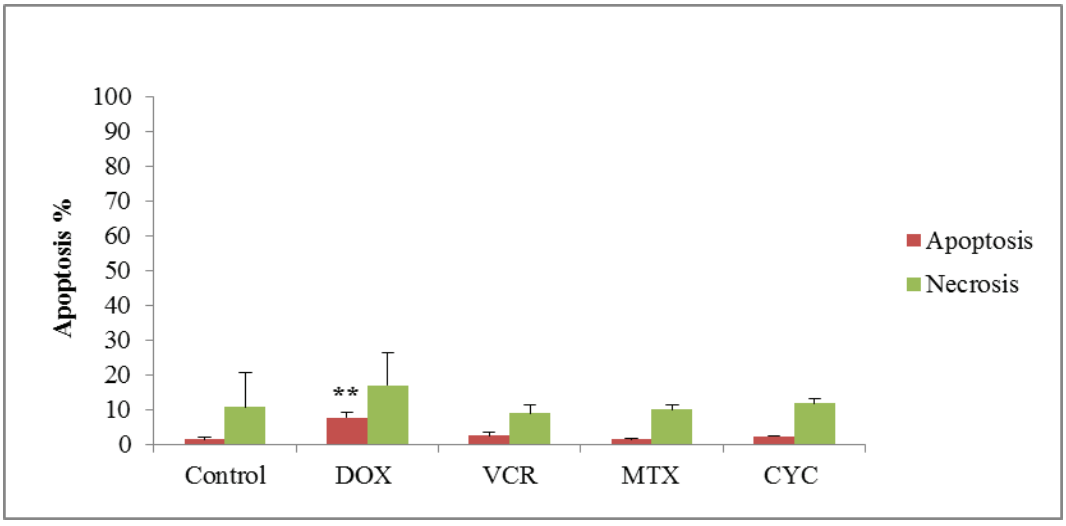
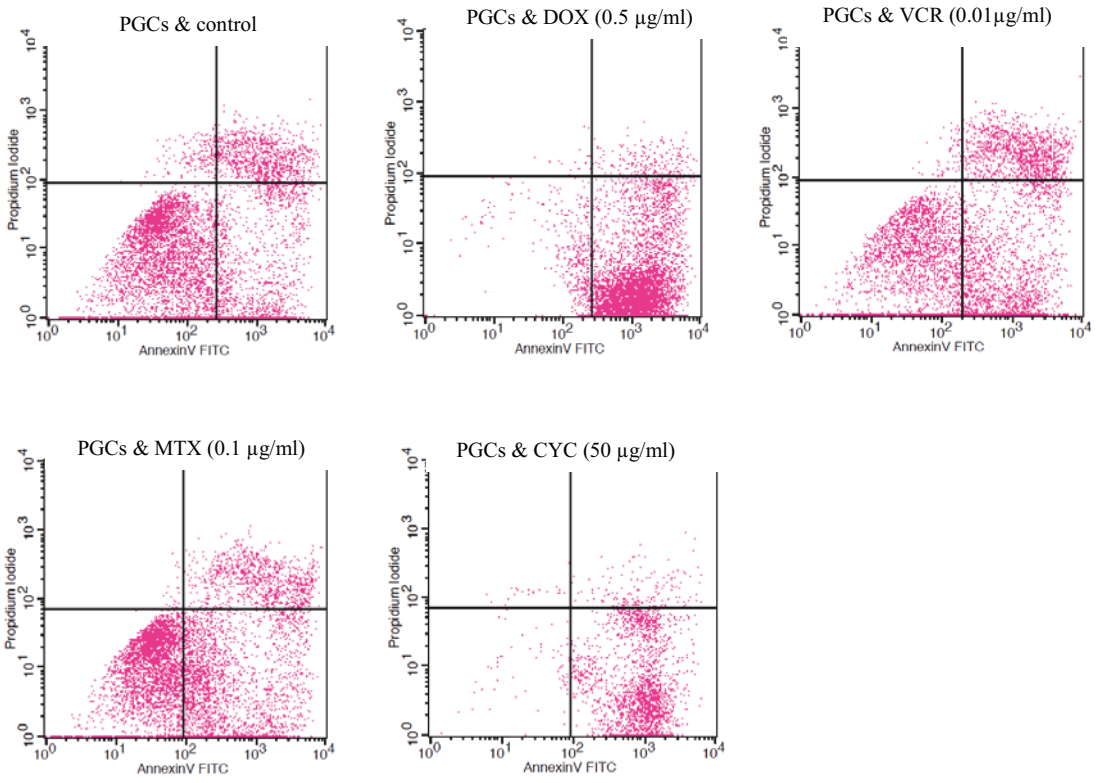


Figure 20. Apoptosis assay by annexin V showing the cell death in KGN cells.
(A) Flow cytometry for apoptosis assay by annexin V showing the cell death in KGN cells untreated, treated with first concentration which inhibited cell viability > 50% of either doxorubicin (DOX; 0.2µg/ml), vincristine (VCR; 0.01 µg/ml), methotrexate (MTX; 0.1 µg/ml) or cyclophosphamide (CYC; 10 µg/ml) for 48 h and stained with annexin V and Propidium Iodide (PI). (B) The relative percentage of apoptosis and necrosis in KGN cells (n=3 independent experiments, Mean ± SD **p < 0.01; Student's t test).

A



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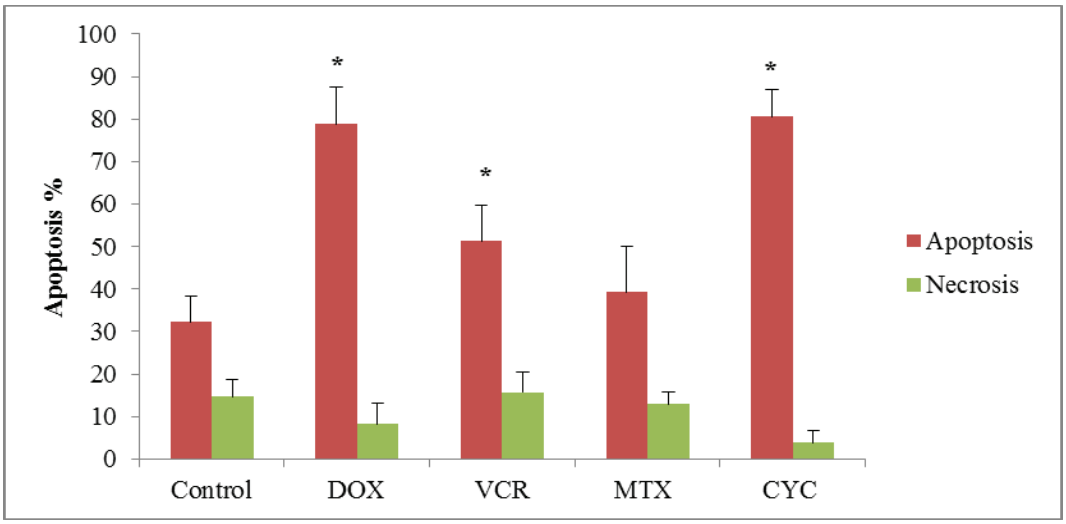


Figure 21. Apoptosis assay by annexin V showing the cell death in primary granulosa cells.
(A) Flow cytometry for apoptosis assay by annexin V showing the cell death in primary granulosa cells untreated, treated with first concentration which inhibited cell viability > 50% of either of doxorubicin (DOX; 0.5µg/ml), vincristine (VCR; 0.01 µg/ml), methotrexate (MTX; 0.1 µg/ml) or cyclophosphamide (CYC; 50 µg/ml) for 48 h and stained with annexin V and Propidium Iodide (PI). (B) The relative percentage of apoptosis and necrosis in primary granulosa cells (n=3- independent experiments, Mean ± SD *p < 0.05; Student's t test).

4.5. Effect chemotherapeutic drugs on expression of microRNAs

To explore the potential effect of the cytostatic drugs on expression of miR-21 and miR-132 in primary granulosa cells and cell lines, cells were treated with (1 µg/ml) of doxorubicin, vincristine, methotrexate or cyclophosphamide for 24 h. Expression was detected by quantitative real time PCR (qRT- PCR) and calculated using the delta delta cycle threshold (ΔΔCt) method relative to the housekeeping small nucleolar RNA U48 (RNU48).

4.5.1 Expression of miR-21 after treatment with cytostatic drugs

Expression of miR-21 was significantly increased in COV434 cells that were treated with doxorubicin but not in KGN (Fig. 22A), primary granulosa cells or Jurkat cells (Fig. 22B), while treatment with vincristine, methotrexate or cyclophosphamide did not significantly change miR-21 expression (Fig. 22).

4.5.2. Expression of miR-132 after treatment with cytostatic drugs

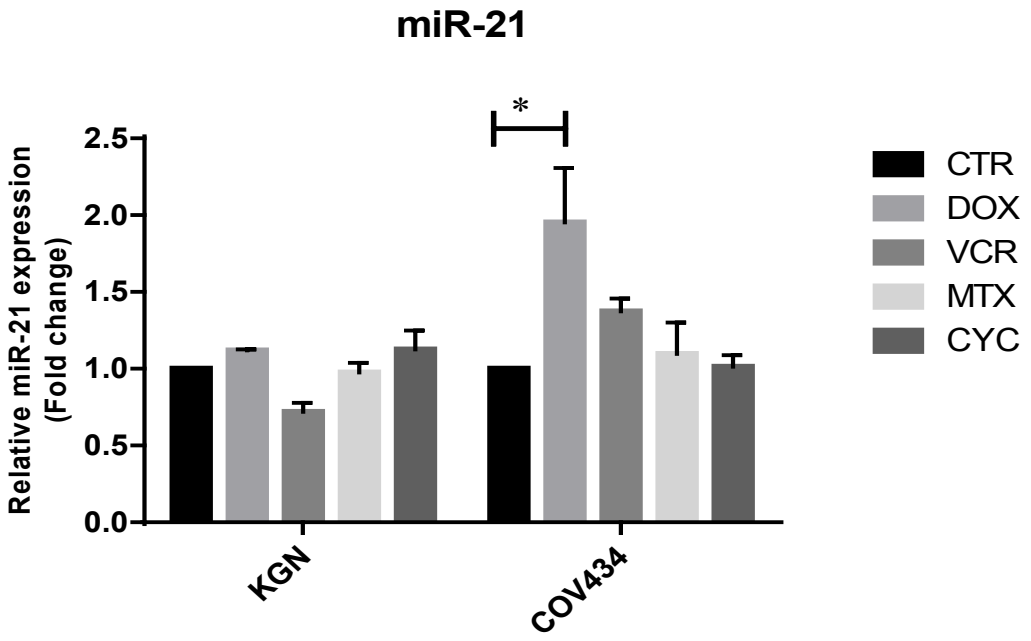
In KGN cells treated with doxorubicin, the expression level of miR-132 was significantly increased (Fig 23A) whereas it was not significantly altered in COV434 cells (Fig 23B). Expression of miR-132 was also significantly increased in Jurkat cells that were treated with doxorubicin or vincristine (Fig. 23B).

4.5.3. Effect of doxorubicin on expression of miR-21 and miR-132 in cells

In order to determine whether the effect of doxorubicin on expression of miR-21 and miR-132 in cells is dose-dependent, KGN and COV434 cells were untreated or treated with increasing doses (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml) of doxorubicin for 24 h.

Expression of miR-21 in COV434 cells (Fig. 24A) and KGN cells (Fig.24 B) was not dose-dependent. In contrast, in COV434 cells, the expression of miR-132 increased in a dose-dependent manner (Fig.25A), but not in KGN cells (Fig.25B).

A



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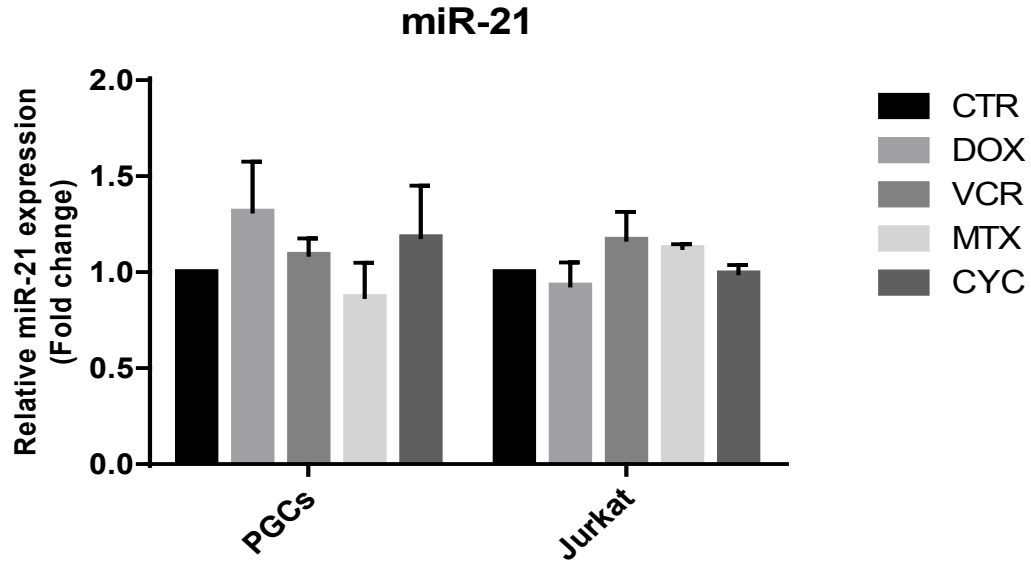
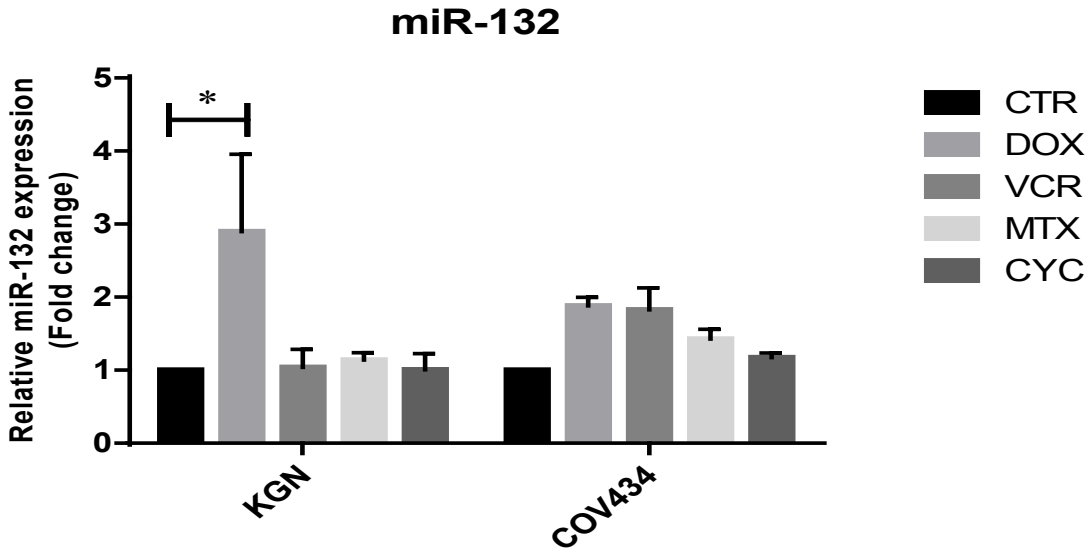


Figure 22. Expression of miR-21 in cells
(A) Relative expression of miR-21 in KGN and COV434 cells, (B) primary granulosa cells (PGCs) and Jurkat cells which were untreated, treated with (1 μg/ml) of doxorubicin, vincristine, methotrexate or cyclophosphamide for 24 h. miR-21 expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping small nucleolar RNA U48 (RNU48). Data are represented as mean ± SE, n = 3 each in triplicates (ANOVA test).

A



B

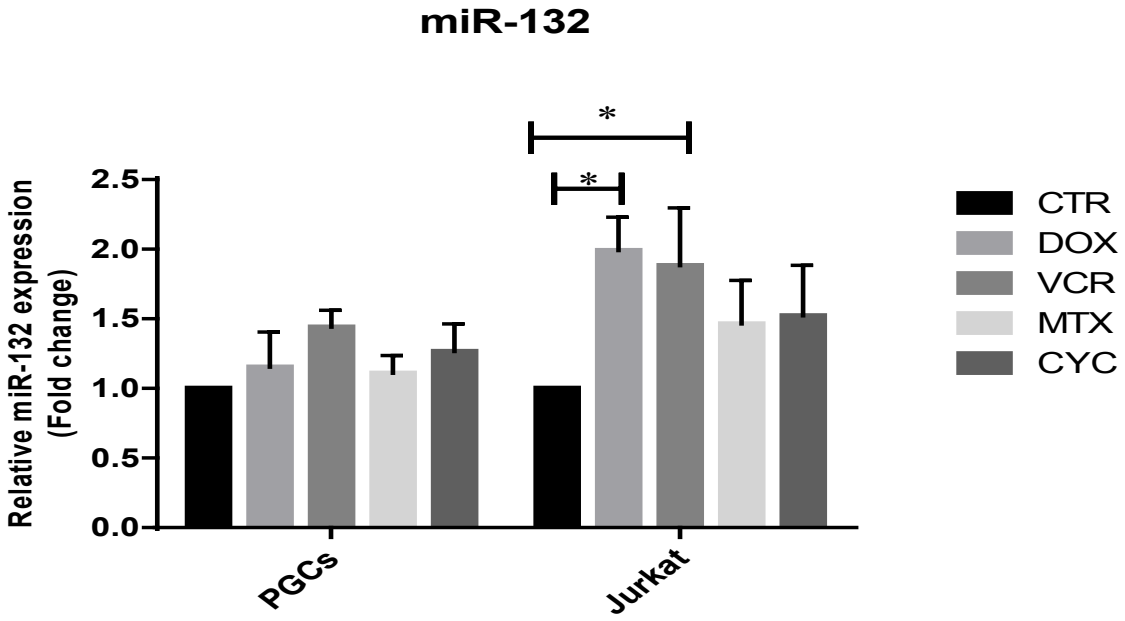
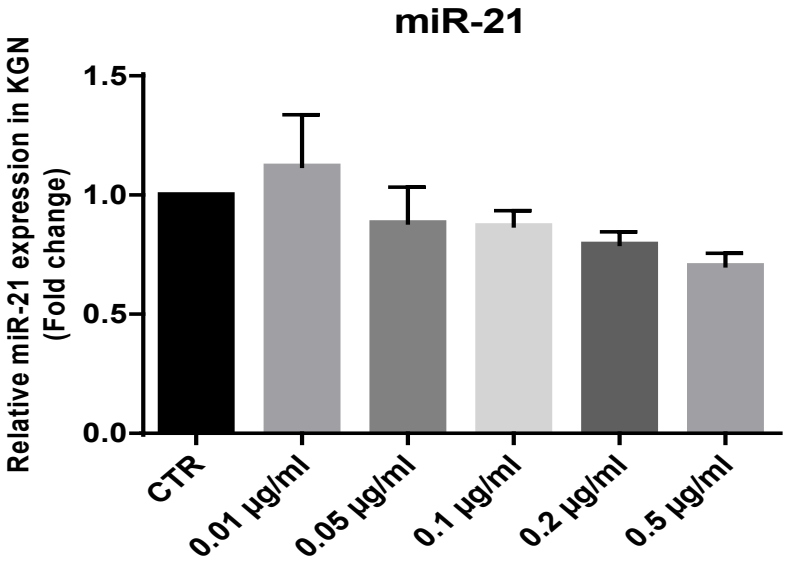


Figure 23. Expression of miR-132 in cells
(A) Relative expression of miR-132 in KGN and COV434 cells, (B) primary granulosa cells (PGCs) and Jurkat cells, which were untreated, treated with (1 μg/ml) of doxorubicin, vincristine, methotrexate or cyclophosphamide for 24 h. miR-132 expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping small nucleolar RNA U48 (RNU48). Data are represented as mean ± SE, n = 3 each in triplicates. *$p < 0.05$ (ANOVA test).

A



B

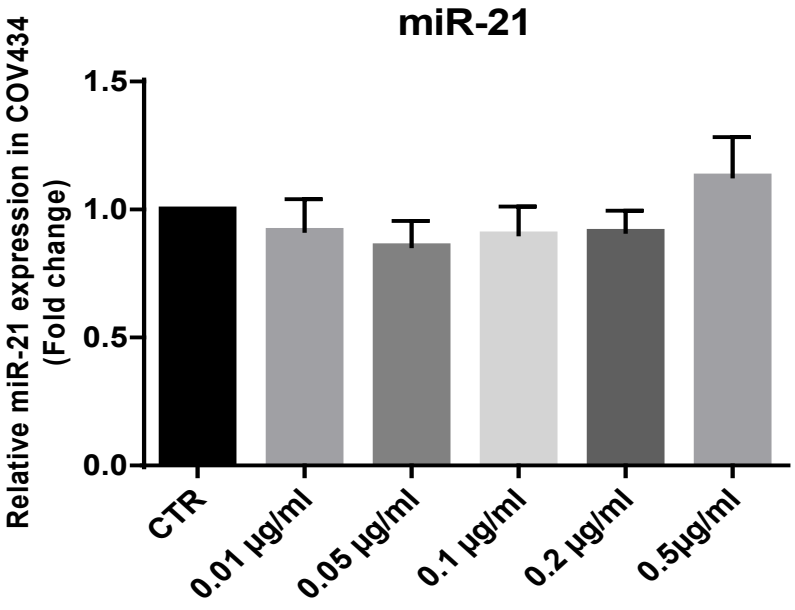
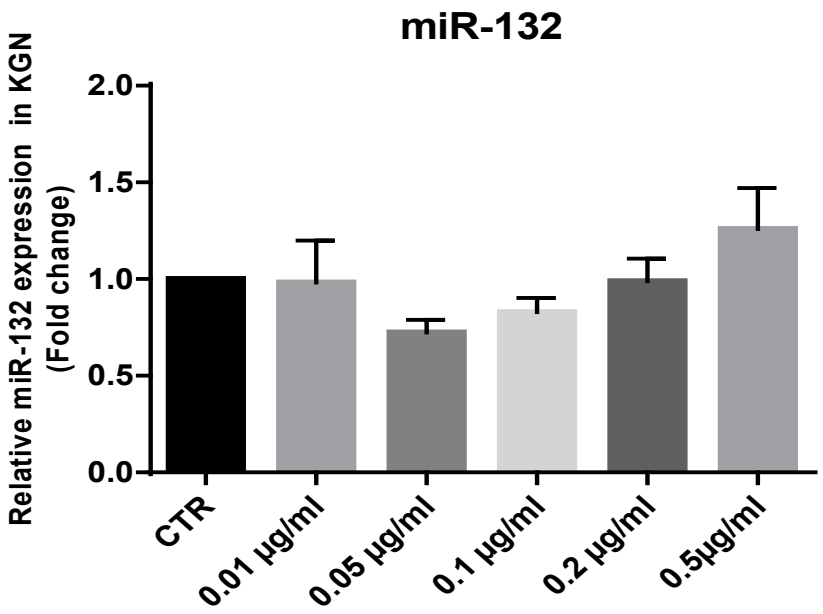


Figure 24. Effect of doxorubicin on expression of miR-21 in cells
Relative expression of miR-21 in (A) KGN cells and (B) COV434 cells were untreated, treated with increasing doses of doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml) for 24h. miR-21 expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping small nucleolar RNA U48 (RNU48). Data are represented as mean ± SE, n = 3 each in triplicates (ANOVA test).

A



B

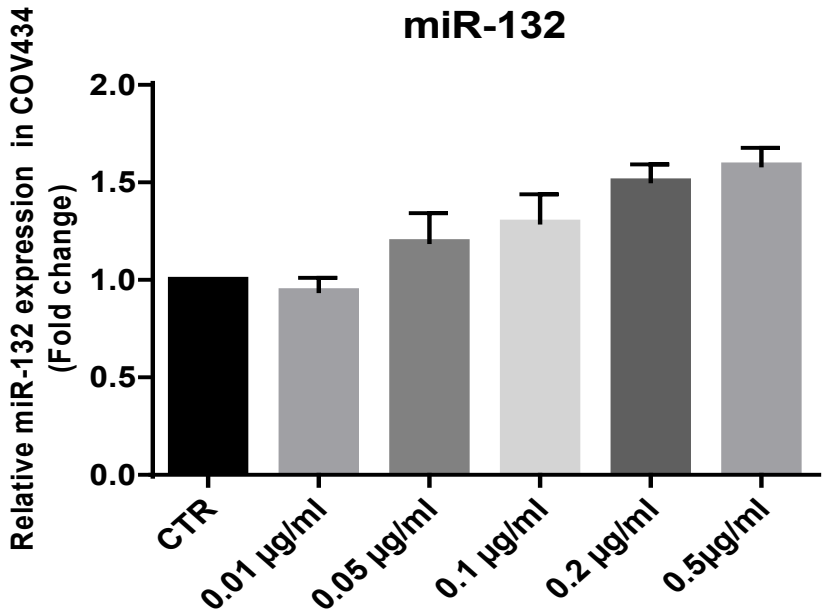


Figure 25. Effect of doxorubicin on expression of miR-132 in cells
Relative expression of miR-132 in (A) KGN cells and (B) COV434 cells were untreated, treated with increasing doses of doxorubicin (0.01, 0.05, 0.1, 0.2, and 0.5 µg/ml) for 24 h. miR-132 expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping small nucleolar RNA U48 (RNU48). Data are represented as mean ± SE, n = 3 each in triplicates (ANOVA test).

CHAPTER 5 DISCUSSION

Childhood cancer is one of the leading causes of death by disease among children in the world and the number of reported new cancer cases is increasing every year. Acute lymphoblastic leukemia is the most common malignancy in childhood, accounting for 26% of pediatric cancer diagnoses (Ward et al. 2014). Aim of childhood cancer treatment is to improve the long term survival rates and maintain healthy life. It has been reported that improvement of childhood cancer treatment has resulted in a 5-year survival rate exceeding 80% (Keegan et al. 2016). Infertility and premature ovarian failure are potential reproductive consequences of exposure to cytotoxic chemotherapy regimens in young females with cancer. The risk of developing premature ovarian failure following chemotherapy has associated risks including cardiovascular disease and psychosocial problems, such as depression (Carter et al. 2005, Jeanes et al. 2007). The rate of infertility in general population is around 5% (Gnoth et al. 2005), in 25 years old women 3% and in 30 years old women 6% (Habbema et al. 2009). Interestingly, the rate of infertility is up to one third higher in survivors of childhood cancer depending on age of patients at treatment and the type of treatment (Balcerek et al. 2012). Therefore, the current strategy is focused on fertility improvement in childhood cancer survivors.

Combination chemotherapy is an essential part of treatment for the majority of childhood cancers and leading to long term remission in childhood ALL (Jenkins 2013). Due to limited data available on the ovarian damage caused by individual chemotherapy agents, this study aimed to investigate the potential effect of different drugs on human ovarian granulosa cells. We selected four different categories of chemotherapy agents (doxorubicin, vincristine, methotrexate and cyclophosphamide) that are commonly used in treatment of childhood cancers with different mechanisms of action and gonadotoxic effect.

The mechanisms of chemotherapeutic agents are not specifically targetting the tumor. They can exert their effects on the growth of many cell types with particular cytotoxicity to dividing cells. Granulosa cells are somatic cells within follicles with a high degree of proliferation, whereas oocytes rapidly develop in follicles, but do not divide. Granulosa cells have their central role in supporting oocyte growth and development as well as in the coordination of folliculogenesis in response to gonadotropin signaling and through their bidirectional communication with the oocyte and theca cells (Knight and Glister 2006).

Moreover, granulosa cells are also involved in production of estrogens via cytochrome P450 aromatase, a key enzyme in estradiol biosynthesis (Albertini et al. 2001, Senthilkumaran et al. 2004). Any impairment of granulosa cells and their ability to proliferate may also have an indirect effect on follicle development and may cause damage of the oocyte and consequently deficient embryo development and poor pregnancy outcome (Morgan et al. 2012). This fact opened the path to study the effect of chemotherapeutics drugs on human granulosa cells.

Despite the inverse relationship between gonadotoxicity of chemotherapy and female age, chemotherapeutic destruction of follicles occurs at all ages (Meirow 2000). Reduction of the likelihood of pregnancy was associated with treatment of alkylating agents in a dose-dependent manner in female survivors from childhood cancer between 1970 – 1986 by Childhood Cancer Survivor Study (CCSS) (Green et al. 2009).

There are no comparisons so far of effects of chemotherapeutic drugs on human leukemia T cell lines and human granulosa cells. Hence, in this study we have analysed and compared the effects of chemotherapeutic drugs on viability and apoptosis of normal primary granulosa cells, the granulosa cell lines KGN, COV434 and human leukemia T cell line Jurkat.

The protocol used in this study allows the isolation of primary granulosa cells from follicular aspiration from patients undergoing IVF or ICSI. Follicular fluid contains mainly luteinizing granulosa cells. Red and white blood cells may be also present in this fluid, which may disturb further experiments if they are not completely removed (Quinn et al. 2006).

Two granulosa cell lines were used in this study: the steroidogenic human ovarian granulosa cell line, KGN, which contains most physiological properties such as expression of FSH receptor and pattern of steroidogenesis similar to normal human granulosa cells (Nishi et al. 2001), and the immortalized granulosa cell line COV434 that also expresses FSH receptor and produces estradiol through the action of cytochrome aromatase and possesses many of characteristics of normal luteinized granulosa cell (Zhang et al. 2000). KGN was isolated from a patient with invasive ovarian granulosa cell carcinoma (Nishi et al. 2001), while COV434 was isolated from a 27 years old female patient with metastatic granulosa cell carcinoma (Vandenbergbakker et al. 1993).

Our tested chemotherapeutic drugs are commonly used in treatment of acute lymphoblastic leukemia in children. Therefore, we used Jurkat cells, an acute T lymphoplastic leukemia cell line of human origin as a model for this study (Abraham and Weiss 2004).

The human risk of chemotherapeutic agents can be categorized according to their ovarian failure risk into three groups: high, medium and low risk (Wallace et al. 2005, Blumenfeld 2012). The risk of impairment of ovarian function in patients treated with alkylating agents is a fourfold higher than in patients treated with other chemotherapy agents (Meirow und Nugent 2001). Cyclophosphamide is an alkylating agent posing the highest risk for ovarian failure in a study more than 40 years ago (Warne et al. 1973). Our preliminary experiments with high concentrations of cyclophosphamide indicated that it was non-cytotoxic in vitro, because it is a pre-drug and requires to be converted to active form in liver (Teicher et al. 1996). Therefore, we decided to use the active metabolite of cyclophosphamide (4-hydroperoxy-cyclophosphamide) which is similar to other studies (Tsai-Turton et al. 2007, Turgeman et al. 2014, Yuksel et al. 2015).

In this study, the cytotoxic effects of each treatment were evaluated by using a methyl tetrazolium salt (MTS) assay that shows the metabolic activity of cells. A slight reduction of cell viability was observed after 12 and 24 h of culture. Therefore, cells were treated for 48 h. Our results demonstrate that treatment with cyclophosphamide for 48 h caused a significant degree of cytotoxicity on all cell lines. The observed reduction in viability of primary granulosa cells by high concentrations of cyclophosphamide (50 and 100 µg/ml) was massive and in line with the observed reduction of estradiol production in cultures treated with same concentrations of this drug. This finding is similar with a result of a recent study (Bildik et al. 2015). Estradiol has been shown to play an important role in follicle development and ovulation.

Staining with fluorescein-conjugated Annexin V and PI demonstrated the percentage of apoptotic and necrotic cells in our study. We found that 50 µg/ml cyclophosphamide treatment induced primary granulosa cell apoptosis. Our result is in agreement with a recent study that reported cyclophosphamide induced apoptosis in human granulosa cells as well as in primordial and growing follicles in the rat ovary (Yuksel et al. 2015). Another study showed that cyclophosphamide decreased functions of human granulosa cell line COV434 in vitro and initiation of apoptosis by inducing oxidative stress mediated by glutathione depletion (Tsai-Turton et al. 2007). Gonadal toxicity with cyclophosphamide alone or in combination with other chemotherapeutic drugs has been documented. The authors found that in women treated with cyclophosphamide and doxorubicin, 84% became amenorrhoeic by the end of treatment (Petrek et al. 2006) and previous studies confirmed that cyclophosphamide has an extremely damaging effect on the ovary (Ataya et al. 1990, Meirow et al. 1999). Primordial follicles were decreased in mice treated with cyclophosphamide in a dose dependent manner (Meirow et al.

1999). Moreover, a single dose of cyclophosphamide induced a severe reduction in human primordial reserve in a xenograft model (Oktem and Oktay 2007b).

Doxorubicin, an anthracyclin antibiotic, causes cell death through intercalation with genomic DNA, inhibition of topoisomerase enzymes, DNA replication and cell division (Jurisicova et al. 2006). It is an essential drug in treatment for many different childhood cancers (Essig et al. 2014). However, risk of doxorubicin on fertility has been categorized as medium (Wallace et al. 2005). It has been linked to premature ovarian failure by inducing ovarian damage and follicle loss (Ben-Aharon et al. 2010, Morgan et al. 2012). As observed in previous studies, in human primordial follicles both the granulosa cells and oocyte were impaired after doxorubicin treatment in vitro (Soleimani et al. 2011, Morgan et al. 2012). In our study we found that there was a significant decrease in viability of all tested cells after treatment with doxorubicin in dose-dependent manner. At the highest dose of doxorubicin (0.5 μg/ml), the percentage of apoptotic cells was 80%. Estradiol expression gradually declined simultaneously along with increasing concentrations of this drug in primary granulosa cell cultures. This finding is consistent with previous reports that doxorubicin affects granulosa cell viability and induces apoptosis (Ben-Aharon et al. 2010, Morgan et al. 2012, Roti et al. 2012, Sanchez et al. 2013, Turgeman et al. 2014). Granulosa cells are mitotically active and their sensitivity to doxorubicin may be related to the fact that doxorubicin interferes with cellular replication and transcription. Therefore, granulosa cells are the main ovarian target of cell damage by doxorubicin which causes indirectly germ cell death, and hence, follicle loss.

To date, in general, the evidence suggests that the dosage of the chemotherapy treatment is an important factor for induction of early menopause (Chiarelli et al. 1999). Our results revealed that cyclophosphamide and doxorubicin dose-dependently induced apoptosis in granulosa cells.

There are too limited data available on the effects of methotrexate on human granulosa cells in vitro. Methotrexate is an antimetabolite and an essential drug in the treatment of acute lymphoblastic leukemia in childhood (Essig et al. 2014). It acts specifically during the S-phase of the cell cycle, where it prevents the growth and proliferation of dividing cancer cells by inhibition of folic acid production, which is required for nucleotide biosynthesis (Rajagopalan et al. 2002). The estimated risk of gonadal dysfunction with methotrexate is low (Wallace et al. 2005). Our results showed that methotrexate was highly effective in inhibiting Jurkat cells viability, whereas primary granulosa cells and cell lines were comparatively resistant to the cytotoxic effects of methotrexate at higher concentrations. Moreover, treatment of primary granulosa cells with different concentrations of methotrexate had no effect on estradiol level. Thus, this finding suggests that methotrexate may be useful as therapeutic agent in treatment of acute lymphoblastic leukemia with less cytotoxic effects on granulosa cells. Our findings are in line with previous studies reporting no effects of methotrexate on fertility in ectopic pregnant women (Oriol et al. 2008, Uyar et al. 2013). Moreover, it has been reported that addition of methotrexate to alkylating agent regimens was not associated with an increase in amenorrhea after treatment. Benian et al. (2013) investigated the effect of methotrexate on rat ovaries by measuring serum AMH, that is produced by granulosa cells in primary to small antral follicles of the adult ovary. There was no change in AMH levels in serum before and after treatment (Benian et al. 2013). In contrast, another study observed reduction of endometrial glands and ovarian follicles in uteri and ovaries of mice, which were treated with methotrexate for 6 months (Chelab and Majeed 2009). In another study, treatment with methotrexate has been associated with early ovarian failure in childhood cancer survivors (Lantinga et al. 2006).

To the best of our knowledge, there are no previous studies in literature on effects of vincristine on human granulosa cells and cell lines in vitro.Vincristine is an antimicrotubule agent, disrupts microtubule structures of the cell cytoskeleton and mitotic spindle which leads to mitotic block at the metaphase and apoptosis (Jordan 2002, Jordan and Wilson 2004). It is effective in the treatment of acute lymphoblastic leukemia (Ong et al. 2008). In our hands, vincristine had the highest cytotoxic effect on the viability of Jurkat cells and both granulosa cell lines (KGN and COV434), but there was no deleterious effect on the viability of primary granulosa cells. This finding may suggest that primary granulosa cells can respond differently to vincristine. One explanation for this difference in response to vincristine between malignant cells (KGN and COV434) and non-malignant cells (primary granulosa cells), is the proliferation capacity of the two cell types: cancer cells proliferate rapidly, whereas primary granulosa cells proliferate minimally or do not proliferate at all (Ferrero et al. 2012).

Interestingly, the percentage of apoptotic and necrotic cells following treatment with vincristine at the lowest concentration (0.01µg/ml) was markedly increased in Jurkat cells when compared to similar concentrations of this drug in the primary granulosa cells and cell lines, which may indicate that Jurkat cells are more susceptible to vincristine-induced cell death. Our results are in agreement with a previous study that vincristine induced cell death in Jurkat cells (Shen et al. 2013). Vincristine, at low concentration, has been reported to be less gonadotoxic. Also our results demonstrated that it had lower toxicity on granulosa cells than cyclophosphamide and doxorubicin with regard to their estradiol production.

Another interesting point is the high sensitivity of Jurkat cells to low doses of all tested chemotherapeutic drugs in our study. The dose-and time dependent cytotoxic effects on these cells are consistent with previous studies (daSilva et al. 1996, Friesen et al. 1996, Gamen et al. 1997, Amit et al. 2009, Sabova et al. 2010).

Development of drugs for protection of non-tumor cells or specific ovary protection may reduce chemotherapy side effects. Several studies have demonstrated potentially beneficial treatments (Imai et al. 2007, Morgan et al. 2012). The best way of protecting the ovary from chemotherapy-induced damage could be to directly and specifically prevent any damage caused.

microRNAs are short non-coding RNA molecules and consist of approximately 21 nucleotides and represent approximately 1–3% of genes in humans (Silveri et al. 2006). miRNAs regulate gene expression of hundreds of target mRNA post-transcriptionally by binding to the mRNA 3'- untranslated region. miRNAs control physiological and pathological processes such as cell proliferation, cell differentiation, apoptosis and tumorigenesis (Lee and Dutta 2009). Recently, miRNAs have begun to be explored in ovary where they have a regulatory role in folliculogenesis, oocyte maturation, implantation and early embryonic development (Hossain et al. 2009, Sirotkin et al. 2009, Donadeu et al. 2012).

It is well documented that miRNAs are expressed in human granulosa cells and follicular fluid. miR-21 and miR-132 are among the most abundantly expressed miRNAs in human oocytes and granulosa cells (Assou et al. 2013, Velthut-Meikas et al. 2013) as well as in human follicular fluid (Sang et al. 2013), suggesting their important roles in ovarian granulosa cell functions. Therefore, based on these findings and existing literature we aimed to investigate effects of chemotherapeutic drugs on miR-21 and miR-132 expression in different granulosa cell lines and primary granulosa cells as well as in Jurkat cells.

Interestingly, our experiments revealed that doxorubicin led to overexpression of miR-21 in COV434 cells but not in KGN cells. By increasing the doxorubicin concentrations, expression of miR-132 in COV434 cells increased. Previous studies revealed that cell proliferation and chemoresistance to doxorubicin were promoted by overexpression of miR-21 in bladder cancer cells (Tao et al. 2011). A recent and functional study has demonstrated that overexpression of miR-21 enhanced cell proliferation, migration and invasion in trophoblast cells (Chaiwangyen et al. 2015). In ovary, miR-21 has been identified as promoting follicular cell survival during ovulation (Donadeu et al. 2012). In Jurkat cells, the level of miR-132 expression increased significantly after treatment with 1 μg/ml doxorubicin and vincristine. In a previous study, a key role of miR-132 in promotion of estradiol synthesis in murine ovarian granulosa cells has been reported (Wu et al. 2015), but its role in human granulosa cells remains unknown. Thus, our future work will continue to elucidate functions of miR-132 in human granulosa cells and to assess its role in the regulation steroidogenesis in these cells.

CHAPTER 6 CONCLUSION

Premature ovarian failure and infertility in adulthood are significant long-term effects of exposure to cytotoxic treatment during childhood. Type and dose of cytotoxic drugs determine the degree of ovarian damage resulting in reduced fertility. Our findings demonstrate that Jurkat cells have a high sensitivity to all chemotherapeutic agents tested in our study which induced dose-and time dependent cytotoxic effects. The results of our study indicate that treatment of primary granulosa cells with doxorubicin and cyclophosphamide results in a significant increase in cell death and a dose-dependent decrease in cell viability and estradiol production. In contrast, there is a comparatively low impact of methotrexate and vincristine on the viability and steroidogenesis of human primary granulosa cells.

Overall, the primary granulosa cells and cell lines express miR-21 and miR-132 which play important roles in cell functions. We demonstrated that doxorubicin strongly enhances expression of miR-21 and miR-132 in granulosa cell lines. It may be argued that similar effects occur in vivo, but their potential influence on fertility cannot yet be estimated.

The knowledge about the potential damaging effects of cytostatic drugs on human ovarian function should be considered in selection of treatment and in development of novel treatment regimens. More research on the cellular mechanisms behind chemotherapy-induced follicle loss may lead to the generation of treatments specifically designed to prevent premature ovarian failure and infertility.

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ACKNOWLEDGMENT

I wish to express my gratitude to all individuals have contributed in making this PhD study process such a pleasant and rewarding experience.

First and foremost, I would like to express my sincere gratitude to my thesis supervisor, Prof. Dr. med. Udo Markert for his excellent guidance, suggestions, encouragement, and understanding which help me deeply throughout the whole research work. I am very much thankful and grateful for the Ph.D. student position in the Placenta Lab.

I would like to extend my appreciation to all Placenta Lab members, especially for Dr.Wittaya Chaiwangyen for teaching me in the lab, advices and all help, Jana Pastuschek for helping and caring and Dr. Andre Schmidt for helping in correction this thesis. Many thanks for Dr. Stella Mary for loving and caring. Deep thanks for Dr. Diana Morales, Julia Heger, Karolin Fröhlich, Christin Bär, Maja Weber and Ruby Gutierrez for helping and supporting.

Many thanks for Dr. med. Andreas Fritzsche and Dr. rer. medic. Simone Winkler, (Center for Fertility Jena and Erfurt) for cooperation and providing the follicular fluid samples.

I would like to express my gratitude to the Ministry of Higher Education and Scientific Research in Yemen and Graduate Academy Friedrich-Schiller University Jena for financial support.

Finally, deep thanks from bottom my heart to my mother for her love, guidance and support to every step of my live and my most sincere and deepest gratitude goes to my beloved Moneeb for all his love, support and all the joy he bring into my life. A special thanks to my brothers and sister and all my big family for all the love and the concern that has provided me all the time. Last, but not least, many thanks for my friends in Yemen: Samia, Asrar, Hend, and in Germany: Aisha, Nasema, Nehal and Lila for loving and all support.

EHRENWÖRTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts durch Korrekturlesen unterstützt haben: Prof. Dr. med. Udo Markert, Dr. Andre Schmidt und Dr. Wittaya Chaiwangyen.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

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Jena, 03.01.2017 Boodor Al-Kawlani.